

TEXTBOOK OF BACTERIOLOGY



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(ELEVENTH EDITION OF MUIR & RITCHIE'S "MANUAL")

BY

C. H. BROWNING, M.D., LL.D., D.P.H., F.R.S.

GARDINER PROFESSOR OF BACTERIOLOGY, UNIVERSITY OF GLASGOW

AND

T. J. MACKIE, C.B.E., M.D., LL.D., D.P.H.

PROFESSOR OF BACTERIOLOGY, UNIVERSITY OF EDINBURGH

Geoffrey Cumberlege

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PREFACE

THE first edition of Muir and Ritchie's 'Manual of Bacteriology' was published fifty-one years ago and it is eleven years since the last, a further edition having been delayed by the circumstances of the War. In this period the number and extent of the advances in almost all branches of the science have been so great that it has proved impossible to preserve the original treatment of the subject and at the same time to retain the old format. Accordingly, the whole book has been recast and set on a larger page, while the proportion of small type in the text has been much reduced, we trust, with advantage to the reader's comfort and convenience. Also, the opportunity has been taken to rewrite as well as to rearrange the matter, so that practically all the chapters are new in greater part. At the same time it has been a chief object to preserve those characters to which the book owed its great popularity. Thus in each section we have aimed at giving a critical survey of knowledge up-to-date, along with an account of the basic information yielded by laboratory and clinical investigations. However, in these days when book-production is subject to much delay, the reader must accept that, since paging of the type began early in 1948, it has not been possible to incorporate many additions to knowledge after that time. While emphasis is laid principally on those micro-organisms responsible for diseases in man, the infections of animals also receive attention, owing to the two being so closely associated. As hitherto, fungi and protozoa are included along with the pathogenic bacteria and viruses, because they all show great similarities in the pathological changes produced, also similar methods are employed for their investigation. We are indebted to Dr. Cranston Low for contributing the chapter on Pathogenic Fungi. The increasing importance of Viruses has necessitated a great extension of the space allotted to these agents. The chapter on Chemotherapy also has been enlarged so as to include a fuller discussion of the biological aspects of the subject. The account of general laboratory methods has been removed to an Appendix. It has been decided to retain this section, because information on the practical work involved in investigating infections is essential for a proper understanding of the results.

The question of bacterial nomenclature and taxonomy has received serious consideration. Of late some bacteriologists have regarded the old and widely used word *Bacillus* to designate any rod-shaped organism, e.g. '*Bacillus typhosus*' or the 'typhoid bacillus', as out-of-date. They would restrict the term to aerobic spore-bearing organisms, while employing *Bacterium* as the name of a group of Gram-negative non-sporing rods, and this usage is prominent in recent British writings. But all Schizomycetes have long been referred to as bacteria, and the science derives its name from the word. Again, American systematists, as represented in 'Bergey's Manual of Determinative Bacteriology', have given much attention to a classification which, so far from being definitive, has undergone considerable alterations in successive editions. Other recent writers of textbooks etc. have been eclectic, but none has produced a wholly acceptable system. Accordingly, at the risk of being considered old-fashioned, we have retained for the most part the nomenclature of previous editions of Muir and Ritchie's 'Manual', which at least leaves the reader in no doubt about

the identity of the organisms referred to. However, in the headings of chapters and sections dealing with particular bacteria, the generic and specific names adopted in 'Bergey' have also been given in most cases.

We acknowledge with gratitude the advice of colleagues in regard to special sections, and would mention particularly Professors D. F. Cappel and A. C. Lendrum, Drs. T. Anderson, W. B. Kyles, I. R. W. Lominski, Janet S. F. Niven and R. D. Stuart, and Mr. J. M. Scouller. Thanks are also due to Dr. K. M. Calver for her very great help in preparing the work for the press and to Dr. M. W. Leckie for her assistance with proofs.

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C. H. B.

T. J. M.

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TEXTBOOK OF BACTERIOLOGY

CHAPTER I

GENERAL MORPHOLOGY AND PHYSIOLOGY OF BACTERIA

MICRO-ORGANISMS were first seen by Leeuwenhoek on examining water, fermented fluids, materials from the mouth, intestine, etc., by means of microscopes which he himself manufactured; and in 1683 he figured different types of bacteria present in material from between the teeth. The fact that some were motile caused them to be accepted as living creatures, but their significance long remained obscure, because it was generally believed that life, at least of the lower kinds, could arise by spontaneous generation from dead matter. It was not until the work of Pasteur that proof could be brought concerning the causal relation of such minute forms of life to the processes of fermentation and putrefaction and of infective diseases. Pasteur demonstrated that spontaneous generation of living micro-organisms does not occur. A simple and conclusive experiment which he performed was the following. Flasks were partially filled with solutions which decompose readily, such as yeast extract and sugar, urine, etc. Then the necks of the flasks were softened by heat and drawn out into narrow curved ends with several bends, but not sealed. Finally the flasks were heated until boiling occurred and steam escaped actively for some minutes. It was found that the contents of these flasks remained unaltered indefinitely although the air had free access to their interior. Accordingly it follows that the presence of living micro-organisms in any material is due to their introduction in the living state and subsequent multiplication under suitable conditions. The micro-organisms which are responsible for the infective diseases of man and animals include primitive forms, both of the plant and animal kingdoms. The causal agents of very many of the infective conditions in countries with temperate climates are bacteria, which are accepted as being among the lowest forms of plant life; and with these we are chiefly concerned. In addition, however, other plant forms, corresponding to the moulds and yeasts, namely, true fungi, and also the lowest members of the animal kingdom, the protozoa, require consideration in view of the occurrence among them of many pathogenic species. Classification of unicellular organisms is difficult, and has been the subject of much controversy. Certain micro-organisms are also difficult to assign to either the plant or animal kingdom. General agreement amongst systematists is, however, now being attained and the principles of classification are becoming stabilised, though there still remain matters of doubt. Further, it is now well recognised that various important and prevalent diseases are due to 'viruses' which are not demonstrable by ordinary methods, owing to their size being below the limit of resolution. Such property of often known as *filterable viruses* or *filter-passers*. These terms have, however,

only a relative significance, as some of the filterable viruses have recently been defined as very minute microscopically visible bodies ; also, a particle may pass through or be retained according to the character of the filter, conditions of filtration, etc. ; further, some organisms which are microscopically visible may pass filters under certain circumstances. The question whether these viruses are living organisms has been a matter of controversy, and constitutes one of the most interesting and fundamental of biological problems. The more important diseases produced by the filterable viruses will also receive consideration.

The bacteria collectively form the class designated *Schizomycetes*. They are unicellular organisms of various forms, most of which are devoid of chlorophyll, and without a nucleus which can be differentiated by ordinary methods, and they multiply in general by simple fission ; some are motile, others are non-motile. They are of comparatively simple structure and in one system of classification have been arranged in two main subdivisions—a lower and simpler, and a higher and more organised. The study of bacteria is greatly aided by the fact that in many cases they can be grown on artificial media and that by this means pure cultures of a single species can be obtained.

The *lower bacteria* or *Eubacteria* are the more numerous, and are minute, relatively undifferentiated masses of protoplasm, which produce similar cells by simple fission. Some are motile, others non-motile. Their minuteness may be judged by the fact that in one direction at least they usually do not measure more than $1\ \mu$ ($\frac{1}{10000}$ millimetre or $\frac{1}{80000}$ inch). These forms can be broadly classified according to their shapes into four main groups—(1) A group in which the shape is approximately globular. A member of this is called a 'coccus'. (2) A group in which the shape is that of a straight rod—the proportion of the length to the breadth of the rod varying greatly among the different members. This form is called a 'bacillus'. (3) A group in which the shape is that of a curved rod ('vibrio') or a non-flexuous spiral filament ('spirillum'). (4) A group of filamentous flexuous organisms showing undulations or true spirality. An organism of this type is designated a 'spirochæte'. There has been much dispute with regard to the classification of this last group. Many of them have been regarded as protozoon-like and belonging to the animal kingdom, they are now, however, generally placed amongst the bacteria. Further, all the spirochætes have been grouped in the American classification as a distinct order of the Schizomycetes co-equal with the Eubacteria (p 45). A detailed description of the characters of those groups will be more conveniently taken later (p 37 *et seq.*). In some cases, especially among the bacillary forms, there may occur under certain circumstances changes in the protoplasm whereby a resting phase or spore is formed. It should be noted that while many species of bacteria are very constant in their microscopic appearances, others show marked differences depending on conditions affecting their environment and the age of the culture. Such pleomorphism, as it has been termed, may be a source of difficulty in the identification of organisms.

The *higher bacteria* consist of filaments made up of simple elements such as occur in the lower forms. These filaments may be more or less septate, may be provided with a sheath, and may show branching, either true or false. The structure of the elements comprising the filaments is analogous to that of the lower forms. Their size, however, is often somewhat greater. The higher bacteria show advance on the lower along two lines. (1) While the lower forms sometimes occur in filaments, every member of the filament is independent, but in the higher forms a certain interdependence among the individual elements is apparent. For instance, growth may occur only

at one end of a filament, the other forming an attachment to some fixed object. (2) The higher forms present this further development, that in certain cases some of the cells are specialised reproductive units.

The relations of the bacteria to the animal kingdom on the one hand and to the plants on the other constitute a difficult question. They may be regarded as a group of small unicellular organisms which may be regarded as the most primitive of the animal kingdom.

algæ, and the bacteria. To the lower algæ the bacteria show many similarities. These algæ are unicellular masses of protoplasm, having generally the same shapes as the bacteria and fungi. They are also capable of fission and multiplication.

From these algæ and the bacteria, and from the fact that fission plays a predominant part in the multiplication of both, they were formerly grouped together in one class as the Schizophyta or splitting plants. Of the two divisions forming these Schizophyta the splitting algæ have been denominated the Schizophycæ, while the bacteria or splitting fungi have been called the Schizomycetes.

GENERAL MORPHOLOGY OF THE BACTERIA

On account of the minute size of bacteria high magnifications are required in order to see details of structure. But mere increase in size is of little value unless there is high resolution, *i.e.* capacity to distinguish closely adjacent points. With the ordinary compound microscope the best optical systems practically do not permit of resolving two points which are under 0.2μ (0.0002 mm) apart, although much smaller objects can be seen. Thus no advantage is gained by magnification beyond about 2,000 diameters. By the use of ultraviolet light points 0.1μ apart may be resolved, this permitting a

electron microscope has also been used. In this apparatus rays of electrons moving at high velocity are concentrated on to the specimen, which must be dry and supported on a very thin film of nitro-cellulose. The electrons which have traversed the object are 'guded' by magnetic fields produced by coils of magnetised wire; these act like the objective and eyepiece lenses of an ordinary microscope. Finally the electrons strike a fluorescent screen or a photographic plate. The whole apparatus works *in vacuo*. The high resolving power of the electron microscope permits magnifications of upwards of 180,000 diameters, but 10,000 is a practical optimum for detail.

Microscopic structure. When examined under the microscope, in their natural condition in a watery medium, bacteria appear merely as fairly rigid colourless refractile bodies of the different shapes named. Spores, inclusions, and motility, when these exist, can also be observed, but little else can be made out. For detailed investigation advantage is taken of the affinity of bacteria for various stains, especially basic aniline dyes which stain intensely the nuclei of cells. Usually bacteria are killed before staining, either by heating a thin film dried on glass or by chemical fixatives. Certain features have thus been determined. The bacterial cell consists of a sharply contoured mass of cytoplasm (or protoplast) which reacts to dyes like the nucleus of an animal or plant cell. Healthy bacteria when thus stained commonly appear as finely granular or almost homogeneous structures, which in older individuals may, however, contain various inclusions. The protoplast is

surrounded by an 'envelope', the details of which are seen when a basic stain, such as crystal violet, is added to the living organisms (*e.g.* *B. subtilis* mounted in 25 per cent. solution of sodium chloride). (1) There is an outer faintly stained 'cell wall' appearing as a halo; it is firm, rigid, and somewhat elastic, and holds together groups of certain organisms, *e.g.* streptococci, diphtheroid bacilli, etc. (2) Then internal to this is a narrow 'cytoplasmic membrane', which is sharply contoured and assumes a deeper tint than that of the enclosed cytoplasm; it is well seen by dark-ground illumination and stains deep brown with iodine. External to the cell wall a 'mucoid' layer may be made out. But certain organisms under suitable conditions of growth produce material which forms a distinct, bulky, sharply defined 'capsule', which does not stain readily; these are known as capsulate bacteria (*vide* Fig 3, Nos 4 and 15). If the capsule is glutinous, then a large number of the organisms may occur in masses embedded in what appears to be a matrix of jelly; this is known as a *zoogloea*. On the other hand, if the capsule does not have this cohesive property the separation of individuals may easily take place, especially in a fluid medium in which they may float entirely free from one another. Also, the capsular material may dissolve in the medium. Some organisms, again, tend to adhere in clumps because, owing to their constituents, their surface is not readily wetted, *e.g.* the tubercle bacillus. A number of pathogenic bacteria produce definite capsules especially when growing in the tissues, and in some, *e.g.* pneumococcus and anthrax bacillus, a relationship has been established between capsule formation and virulence. In several cases, as in the pneumococcus, carbohydrate materials have been shown to be important constituents of the capsule; in others, such as the anthrax bacillus, the capsular material is protein-like. Many of the higher bacteria possess a sheath which has a much more definite structure than is found among the lower forms. It markedly resists external influences, possesses elasticity, and serves to bind the elements of the organism together. In certain forms the sheath contains granules of iron oxide or other substances.

Staining. The protoplasm of bacteria reacts to stains in a manner similar to chromatin, although the affinity for dyes may vary considerably. Thus the most useful stains are the basic aniline dyes; these have the composition of salts, the basic component of the molecule being that which imparts the colour, *e.g.* in rosaniline acetate. But certain acid dyes, which are compounds of a dye-acid with a base, such as sodium, also stain bacteria, *e.g.* eosin and rose bengal. *Gram's method* of staining is of great value, because by its use organisms can be classified into two groups—one which retains the stain (Gram-positive) and one which becomes decolorised (Gram-negative). The method consists in staining with the triphenylmethane dye, crystal violet (hexamethyl violet)—gentian violet is an analogue—treating the stained preparation with a solution of iodine in aqueous potassium iodide (or a strong solution of sodium chloride); and then applying an organic solvent, such as alcohol, acetone, or a mixture of aniline with xylol. The result is that Gram-positive organisms appear of an intense purple-black colour, whereas Gram-negative ones are decolorised and can be demonstrated by subsequent counterstaining with another dye of contrasting colour, *e.g.* basic fuchsin. Gram-positive organisms vary in their resistance to decolorising, and in some of them only parts of the protoplasm retain the stain, also old and degenerate forms tend to be Gram-negative. Gram-staining appears to depend on the retention of the dye-complex by magnesium ribonucleate in the cytoplasmic membrane. This substance can be removed by enzymes or by treatment with a bile salt in the presence of

oxygen. Provided the treated organisms are kept in a reducing medium, *e.g.* weak formalin, they will take up the ribonucleate again, so that the Gram-positive condition is restored (Henry and Stacey). Whereas Gram-negative organisms are digested by trypsin after they have been heated at 80° to 100° C., similarly heated Gram-positive organisms are not attacked, except the pneumococcus (Kruse).

Acid-fastness is exhibited by the tubercle bacillus and certain other organisms, which are resistant to staining with simple watery dye solutions. A basic triphenylmethane dye, such as basic fuchsin or crystal violet, is used in concentrated solution along with phenol as a penetrating agent. After prolonged exposure to the stain in the cold or a few minutes at temperatures approaching boiling, these organisms and all other materials are intensely coloured, then a strong solution of mineral acid is applied—20 per cent. of concentrated sulphuric, nitric, or hydrochloric acid in water—followed by washing with water, which removes the dye from everything except the acid-fast structures. The property of acid-fastness appears to depend both on the texture of the organisms and on certain chemical constituents (*vide* Chapter VIII).



FIG. 1. Film of a young (non-spored) culture of *B. subtilis* stained to demonstrate nuclear bodies by Robinow's method $\times 5,000$ (From a preparation by Dr J. P. Duguid.)

The question of a nucleus in bacteria The minute size of the bacterial cell hinders accurate observations on any contained structures. Several views have been held, namely that (a) bacteria are all nucleus, or at least that their nuclear material is diffused throughout the protoplasm, because they tend to stain intensely with dyes which have an affinity for chromatin, (b) they possess no nucleus, or (c) nuclear material exists in the form of a thread-like single chromosome or of granules which exhibit varying forms at different stages of growth. The fact that bacteria give a positive Feulgen reaction shows that they possess chemical constituents found only in the nuclei of other forms of living cells—nucleic acids. Robinow has extended earlier observations by staining with Giemsa's solution after treating the preparation with dilute hydrochloric acid or by Feulgen's procedure. He considers that in young cultures of various organisms chromosome-like structures exist of a size sufficient to be demonstrated clearly as a single short dumb-bell-shaped rod which multiplies by lengthwise splitting in a plane at right angles to the long axis of the organism, so that one or several pairs may be present (Fig 1). These findings require to be reconciled with the observation that organisms which have been cut in two by microdissection methods cease to multiply (Wamoscher). The electron microscope has recently demonstrated in bacteria structures interpreted as nuclei.

Cell inclusions. After bacterial cultures have passed the stage of active multiplication various inclusions tend to appear in the protoplasm of the organisms. *Fat*, in the form of one or several refractile granules which do not stain with the ordinary bacterial stains, may be mistaken for spores. The granules are coloured by dyes with an affinity for fat, especially the Sudan stains (Sudan black B, etc.). Fat globules may either be food reserves or products of degeneration. *Volutin* granules (Babes-Ernst or Neisser granules) stain intensely with weak solutions of dyes such as crystal violet or methylene blue and on treatment with polychrome solutions of the latter they show an intense purple colour—metachromatic staining. They are conspicuous in certain species of organisms, e.g. the diphtheria bacillus, on suitable media, particularly after the stage of active growth; later they may disappear again from the culture. The granules of unfixed organisms are readily dissolved by water above 80° C. or by alkalis or 5 per cent. sulphuric acid, but they become insoluble after fixation. They are to be regarded as a storage material of the nature of nucleoprotein. According to Gróh, however, the granules of diphtheria bacilli are living units; and after disrupting the cell in which they have developed, they give rise to rod forms.

Glycogen granules, which stain brown with iodine, may occur and also *starch* (*granulose*, *ogen*) granules which stain blue; both appear to be food reserves.

Flagella and Motility. As has been stated, many bacteria are motile. Motility can be studied by means of hanging-drop preparations. The movements are of a darting, rolling, or vibratile character; they lead to definite translation, different individuals going in distinct directions, and so are distinguished from Brownian movements and those due to currents. The degree of motility depends on the species, the temperature, the age of the growth, and on the medium in which the bacteria are growing. Sometimes the movement is most active just after the cell has divided, sometimes it goes on all through the life of the bacteria, sometimes it ceases, e.g. when sporulation is about to occur. Motility of lower bacteria is associated generally with the possession of fine wavy thread-like appendages called flagella, which for their demonstration require the application of special staining methods depending on the use of a mordant; this leads to a deposition of dye or metallic silver (*vide* Fig. 3, Nos. 13 and 14 and Fig. 4, No. 2). They may be seen in living organisms by dark-ground illumination with a very intense light. Flagella have also been demonstrated with the electron microscope. They have been shown to occur in many bacteria and spirilla, but only in a few species of cocci. They vary in length, but may be several times the length of the organism, and may be at one or both extremities (polar) or all round (peritrichous). When polar they may occur singly or there may be several; in some spirilla a tuft of terminal flagella is present. Flagella may be regarded as spirals which are directed backwards and rotate in the opposite direction to the body of the organism, thus conferring on it a forward movement, thus they would act both as propeller and rudder (Pijper). They readily become detached, and complicated spiral tresses of detached flagella may be found in bacterial cultures. The development and activity of flagella are stimulated by cultivation on 'semi-solid' media. The nature of flagella has been much disputed. In all probability they are derived from the cytoplasmic membrane of the cell, and studies of bacterial antigens have shown that flagella possess antigenic constituents which are different from those of the body of the organism (*vide* p. 102). It must be recognised, however, that not all cases of motility among the bacteria are dependent on the possession of flagella, for among the spirochaetes the movements, which

are of various kinds, are apparently due to contractions of the protoplasm of the cell itself. Among most of the higher bacteria, motility is of similar nature. Recently Pijper has concluded from his observations, particularly on organisms suspended in viscous solutions of methyl cellulose, that in the lower bacteria also motility depends on wave-like spiral contractions of the cytoplasm (protoplasm). Hence the appearance of flagella might be regarded as the effect of movement on the slime (mucoid) layer of the organisms and not as the cause of the motion; but this is not generally accepted.

Reproduction among the Lower Bacteria. When a bacterial cell is placed in favourable surroundings, *e.g.* a suitable culture medium, it multiplies by simple fission. In the process a constriction is stated to appear in the middle while a transverse unstained line develops across the protoplasm at that point. The process goes on till two individuals can be recognised, which may remain for a time attached to one another, or become separate, according to the character of the envelope, as already explained. In most bacteria growth and multiplication go on with great rapidity. Bacteria may reach maturity and divide in from twenty minutes to half an hour. If division takes place only every hour, from one individual after twenty-four hours 17,000,000 similar individuals will be produced. In some cases the bacterial cell enlarges before division, in others the cell divides and each element then expands to its adult size. If, in the latter case, multiplication is proceeding rapidly, great variation in the size of the individuals may be observed. As shown by the results of artificial cultivation, certain bacteria, *e.g.* *B. tuberculosis*, multiply much more slowly. It has been stated that what appears to be a single rod-form may really consist of several individuals, since multiple divisions of the cytoplasm have occurred before obvious fission. Among the spirochaetes longitudinal as well as transverse division has been described, though this is doubtful.

In some cases division occurs in an irregular manner. Thus among the bacilli branched Y-shaped structures may develop and new individuals may be formed at each branch ('three-point multiplication'). Sometimes a constriction forms near the end of a bacillus and a spheroidal segment is separated off, such aberrant elements, however, may not develop further. Unequal division may also result among the cocci, giving rise to rod-shaped forms.

When a small number of bacteria are brought into fresh medium the immediate course of events depends on the state of the culture which furnished the inoculum. If the culture is an old one in which active multiplication of organisms has ceased, then on transference to the new medium reproduction does not start at once; instead there is a period of delay, the 'lag phase', before rapid multiplication starts. This is followed by a period of maximal growth in which the number of cells increases in geometric progression with the time ('logarithmic phase'). Thereafter the rate of division slows down and finally becomes negligible, ultimately the culture may die. When the inoculum is derived from a culture in the phase of maximal growth, there is no lag on transference to fresh medium. Accordingly, the lag phase may be interpreted as evidence that organisms in an old culture have passed into a condition of depressed vitality.

It must be noted that when bacteria are distributed on the surface or in the substance of a solid medium, the resulting growth develops in the form of separate 'colonies'; these reach their greatest size when the organisms are to begin with sparsely distributed, and the colonies are then usually visible to the naked eye. Furthermore, such colonies frequently present appearances which are characteristic of bacterial groups or species (see various illustrations

in later chapters). But while a given bacterial species tends to produce a normal type of colony, a wide range of variation from this may occur. Such variations are generally associated with changes of other kinds (*vide* p. 24).

From investigations by Graham-Smith and others, it appears that the consistence of the envelope (cell-wall) may have an importance in modifying the naked-eye and low-power appearances presented by bacterial colonies, which constitute a feature in the identification of species. Graham-Smith has differentiated four groups—a 'loop-forming', in which the envelope is so tough that, after division, rupture but rarely occurs (*anthrax bacillus*); a 'folding' group, in which the envelope is so flexible and extensible that the members of a chain can be folded on one another as successive divisions take place (*plague bacillus*); a 'snapping' group, in which partial rupture of the envelope occurs on division (*diphtheria bacillus*); and a 'slipping' group, where the envelope readily breaks, and successively developed bacteria slip past each other (*typhoid bacillus*, *cholera vibrio*).

When bacteria are placed in unfavourable conditions as regards food, etc., growth and multiplication take place with difficulty. In the great majority of cases this is manifested by changes in the appearances of the protoplasm. Instead of its maintaining the regularity of shape seen in normal bacteria, various aberrant appearances are presented. This occurs especially in the rod-shaped varieties, in which flask-shaped or dumb-bell-shaped individuals may be observed. The regularity in structure and size is quite lost. The appearance of the protoplasm also is often altered. Instead of, as formerly, staining well, it does not stain readily, and may have a uniformly pale homogeneous appearance, while in an old culture only a small proportion of the bacteria may stain at all. Sometimes, on the other hand, degenerate bacteria contain intensely stained granules or globules which may be of large size. Such appearances are referred to as *involution forms* (Fig 4, Nos 3 and 4). That these forms really betoken degenerative changes (in part at least of autolytic nature, *vide* p. 22) is shown by the fact that on their being again transferred to favourable conditions, only slight growth at first takes place. Many individuals have undoubtedly died, and the remainder which live and develop into typical forms may sometimes have lost some of the original properties of the strain. The question of aberrant forms is discussed also in connection with Variation.

While it is generally accepted that fission is the sole mode of reproduction among the lower bacteria, there have also been described budding and the formation of granules (gonidia) within the cells, which subsequently become free; these granules may be surrounded by a resistant layer—arthrospores. Appearances interpreted as strongly suggestive of gonidium formation in *B. radicicola* have been criticised as merely due to ageing and degeneration. Certain observers have described the occurrence of life cycles (cyclogeny) including sexual reproduction by conjugation, and 'sympasm' formation; in the latter the organisms lose their individual form and fuse into masses from which later new cells arise. These views seem to depend on the interpretation attached to appearances which have been described above as involution forms, no definite proofs of such cycles have been brought forward so far.

There is no conclusive evidence that the ordinary bacteria, *e.g.* the tubercle bacillus, can exist in a phase of minute filterable forms which are capable of developing into the typical forms again, although this is true of the organisms of the pleuropneumonia group.

The reproductive processes among the higher bacteria are referred to later.

Spore Formation. In some species of the lower bacteria, under certain circumstances, changes take place in the protoplasm which result in the formation of bodies called spores, to which the vital activities of the original bacteria are transferred. Spore formation occurs chiefly among the bacilli. Its development in an organism, *e.g.* *Bacillus megatherium*, is indicated by the appearance in the protoplasm in unstained preparations of a clear area which becomes dense and refractile and assumes a cylindrical form and later is spherical; finally in the course of a few minutes it contracts to an ellipsoid (Bayne-Jones and Petrilli). On staining with a dilute solution of basic fuchsin the clear area is more darkly coloured than the rest of the protoplasm before spore formation is complete. Spores may assume a round, oval, or short rod-shaped form, always shorter but often broader than the original organism. In the process of spore formation the rest of the bacterial protoplasm may remain unchanged in appearance and staining power for a considerable time (*e.g.* tetanus bacillus), or, on the other hand, it may soon lose its power of staining and ultimately disappear (*e.g.* anthrax bacillus). This method of spore formation is called 'endogenous', and the spores are known as *endospores*. Bacterial spores are always non-motile. The spore may finally be situated in the centre of the organism (central), or it may be at one extremity (terminal), or a short distance from one extremity (subterminal) (Fig 3, Nos 17-20). In different species the shape and position of the fully developed spores tend to be constant. In structure the spore consists apparently of a mass of protoplasm surrounded by a dense membrane or capsule. When completely developed it does not stain with simple watery dye solutions, but can be demonstrated by the prolonged application of a powerful stain followed by a decolorising agent, which removes the stain from the vegetative parts of the organism. The presence in the spore of a stainable nucleus has been described (Robinow). The all-important property of a bacterial spore is its high degree of resistance to external influences such as heat, drying, chemical agents, etc. Koch, for instance, in one series of experiments, found that while the anthrax bacillus in the unspored form was killed by a two minutes' exposure to 1 per cent carbolic acid, spores of the same organism resisted an exposure of from one to fifteen days. Such resistance has been ascribed to the capsule and to the very small amount of water in the

tail
its
difference in the resistance of spores of different species, also spores of the same species may vary to a marked degree in this respect among themselves.

When a spore is placed in suitable surroundings for the vegetative growth of the organism, it 'germinates', often within a few minutes, and again assumes the original bacillary form. The capsule may dehisce either longitudinally, or terminally, or transversely. In the last case the dehiscence may be partial, and the new individual may remain for a time attached by its ends to the hinged spore-case, or the dehiscence may be complete and the bacillus grow with a cap at each end consisting of half the spore-case. Sometimes the spore-case does not dehisce, but is simply absorbed by the

organisms may, on germination after keeping for many years, give rise to virulent cultures. Bacteriophage also is carried over in spores.

It is important to note that in the bacteria, spore formation is rarely, if ever, to be considered as a method of multiplication. In at least the great

majority of cases only one spore is formed from one organism, and only one organism in the first instance from one spore. Sporulation is to be looked upon as a resting phase of bacteria in contrast with the stage when active multiplication takes place, the latter being referred to as the vegetative phase. Regarding the significance of spore formation in bacteria, there has been some difference of opinion. According to one view, it may be regarded as representing the highest stage in their vital activity. There is thus an alternation between the vegetative and spore stage, the occurrence of the latter being necessary to the maintenance of the species in its greatest vitality. Such a rejuvenescence, as it were, through sporulation, is known in many algae. In support of this view there are certain facts. In many cases, for instance, spore formation only occurs at temperatures specially favourable for growth and multiplication. There is often a temperature below which, while vegetative growth still takes place, sporulation will not occur; and in the case of the anthrax bacillus, if the organism be kept at a temperature above the limit at which it grows best, not only are no spores formed, but the strain may lose the power of sporulation. Further, in the case of bacteria preferring the presence of oxygen for their growth, an abundant supply of this gas may favour sporulation. It is probable that even among bacteria preferring the absence of oxygen for vegetative growth, the presence of oxygen favours sporulation. The second view with regard to sporulation is that bacteria only form spores when conditions, especially food supplies, become unfavourable for vegetative growth, then they remain in this state until placed in more suitable surroundings. Such an occurrence would be analogous to the encystment which occurs under similar conditions in many of the protozoa. Often sporulation can be prevented from taking place for an indefinite time if bacteria are constantly supplied with fresh food (other conditions remaining unchanged). However, the accumulation of substances produced by the bacteria themselves plays a more important part in making the surroundings unfavourable than the mere exhaustion of the food supply. This would explain how spores may appear rapidly in a culture which is multiplying actively. A living spore will always develop into a vegetative form if given a fresh food supply. The spore antigen is distinct from those of the vegetative phase of the corresponding bacilli (Howie and Cruickshank).

THE CHEMICAL COMPOSITION OF BACTERIA

The vegetative forms of bacteria contain a large proportion of water, which averages 75 to 85 per cent. The solid constituents show great variations, depending not only on the species under investigation but also on the composition of the culture media, the temperature of growth, and the age of culture, this applies particularly to the inorganic constituents. The proteins of the bacterial cells are composed of the same amino-acids as those of higher forms of life, they include the phosphorus-containing nucleoproteins (compounds of ribonucleic and desoxyribonucleic acids). Nucleic acids make

up 20 per cent. of the dry weight of hæmolytic streptococci. There are also other protein constituents regarding which, however, little is known. Granules formed by certain organisms, which show metachromatic staining (volutin granules), are believed to consist of reserve stuffs of protein nature. The total nitrogen amounts on the average to 8 to 15 per cent. of the dry weight. Fats, lipoids (lecithin and cholesterol, etc.), and waxes form a considerable proportion of bacterial protoplasm, these are specially abundant in the tubercle bacillus and other acid-fast organisms. The influence of the medium in regard to such constituents is well shown by the finding of Eckstein and Soule that *B. coli* grown on a synthetic medium of which alanine was the nitrogenous constituent, contained considerable amounts of phospholipoids, whereas when cystine was substituted only a trace of these was formed. Fat may be intimately incorporated with the bacterial protoplasm, so that extraction methods are required for its demonstration, or at times it may be present as globules stainable by histological reagents for fats (p. 6). Various carbohydrates have been isolated from bacteria, and some capsules are specially rich in such substances, which are of the nature of hemicelluloses, but cellulose is seldom present. A nitrogen-containing derivative of a carbohydrate allied to chitin is found, and the rigidity of bacteria has been attributed to its presence in the cell wall. Mucin, a compound of protein with carbohydrate, is also formed. Granules of glycogen, which are stained brown by iodine, are sometimes found in bacteria, also granulose (iogen), which is closely related to starch. The inorganic constituents include sodium, potassium, magnesium, calcium, and iron salts, as well as traces of other bases, also phosphorus, which is always in considerable amount, sulphur, and chlorine. According to the findings of Guillemin and Larson the greater part of the diffuse out entirely,

Pigments are produced by many bacteria, although this is not a marked feature in most of the pathogenic organisms. The pigment may be intracellular or extracellular, but usually it is impossible to determine whether the pigment is wholly inside or outside the protoplasm. Often the coloration extends out into the medium for a considerable distance beyond the organisms. Many factors influence pigment formation, thus oxygen is often essential, also suitable conditions as to temperature are required, since an organism which forms pigment at room temperature may fail to do so at 37° C. The composition of the nutrient medium may also be important. Exposure to light tends to intensify pigmentation. Some of the pigments are water-soluble, others are soluble in fat solvents such as chloroform, alcohol, and ether. Certain of the latter are yellow pigments allied apparently to carotene, the pigment which is widely distributed in both animals and plants and gives the colour to serum and egg yolk. Pyocyanin, a blue pigment formed by *B. pyocyaneus*, is a phenazine dye which readily undergoes reversible reduction. The chemical composition of bacterial pigments is, however, still to a great extent obscure, but the bacteriochlorin of purple and green sulphur bacteria is apparently related to one form of chlorophyll. A number of organisms form several pigments. Bacteriochlorin is responsible for the photo-assimilation of the organisms containing it, but in general the function of bacterial pigments is unknown. The respiratory pigments are dealt with later.

Enzymes and toxins are important products to which the action of bacteria is in very great part due, they may either pass into the medium in which the organisms are growing or they may remain intimately associated

with the bodies of the bacteria, becoming free only on the disintegration of the latter. Their properties and actions are dealt with later.

Many bacteria form components of the vitamin B complex, but vitamins A, C, and D are not produced.

THE PHYSICAL CHARACTERS OF BACTERIA

The superficial layers of bacteria constitute a definite envelope to which the organisms owe their shape and which tends to act as a semipermeable membrane, so that osmotic phenomena occur. Accordingly, plasmolysis may take place in hypertonic solutions; and in hypotonic the cells may burst, the protoplasm being extruded at one end as a spherical mass (plasmoptysis).

from changing the salt-content of the medium either in the direction of decrease or increase (Dunlop and Maitland). Since bacteria are largely composed of hydrophilic colloids, they exhibit the behaviour of such colloid matter in general. In virtue of their minute size also, they present a surface which is large, relative to their volume; therefore they are well adapted to take part in adsorption reactions. In weak solutions of sodium chloride most bacterial cells bear a negative electric charge—that is, they show cataphoresis and migrate to the anode when a direct electric current is passed through the fluid in which they are suspended. By measuring the rate of their migration under the influence of a known electric field, the potential difference between the cells and the fluid may be estimated. The uniform suspension which many species of organisms form in water or weak saline, results from the mutual repulsion of the similarly charged particles, while the molecules of the fluid impart to them active Brownian movements. Under the influence of acids, salts, and certain dyes such bacterial suspensions may exhibit agglutination. Also, a culture of organisms may alter spontaneously in its behaviour and become agglutinated by concentrations of salt which previously did not affect it, this phenomenon is usually accompanied by other alterations, such as the change from a 'smooth' to a 'rough' type of colony.

Conditions affecting the movements of bacteria. In some cases differences are observed in the behaviour of motile bacteria, contemporaneous with changes in their life-history. Thus, in the case of *B. subtilis*, movement ceases when sporulation is about to take place. On the other hand, in the bacillus of symptomatic anthrax (*B. chauvæi*), movement continues while sporulation is progressing. Under ordinary circumstances motile bacteria appear not to be constantly moving, but occasionally to rest; the movements become more active if the temperature be raised. In certain species of bacteria which are typically motile, e.g. *B. typhosus*, non-motile strains may exist, this is not invariably associated with the absence of the flagella, however. Motility can be induced in such flagellated cultures by growth of the organisms on semi-solid media. Most interest attaches to the fact that bacilli may be attracted to certain substances and repelled by others. Schenk, for instance, observed that motile bacteria were attracted to a warm point in a way which did not occur when the bacteria were dead and therefore subject only to physical conditions. Important observations have been made on the attraction and repulsion exercised on bacteria by chemical agents, which have been denominated respectively *positive* and *negative chemotaxis* (Pfeiffer). A fine capillary tube filled with the agent and closed

at one end, was introduced into a drop of fluid containing the bacteria under a cover-glass, and the effect was watched under the microscope. The general result was to indicate that motile bacteria may be either attracted or repelled by the fluid in the tube. The effect of a given fluid differs in the case of different organisms, and a fluid chemotactic for one organism may not act on another. Degree of concentration is important, but the nature of the fluid is more so. Of inorganic substances, salts of potassium are the most powerfully attracting. Further, solutions of peptone and meat-extract have powerful attracting properties. Carbohydrates in solution have little effect, and glycerol is neutral. Acids, alkalis, and alcohol are repellent. Corresponding chemotactic phenomena are shown also by certain animal cells, *e.g.* leucocytes, to which reference is made later.

GENERAL PHYSIOLOGY OF THE BACTERIA

There are six prime factors in the life and growth of bacteria which must be considered, namely, food supply, moisture, relation to gaseous environment, temperature, hydrogen-ion concentration, and light. It should be noted that no other class of creatures shows such a wide diversity of living conditions as the bacteria.

Food Supply and Metabolism. In nature bacteria live chiefly on the complex organic substances which are derived from plants and animals or which constitute their dead bodies. As a general rule, many varieties of bacteria grow side by side, so that the food supply of any particular one may depend on the growth of others. But while such *symbiosis* may favour some species, certain organisms are inhibited by the presence of others (*antibiosis*). The production of a disease, however, is usually due to the penetration of one species into the tissues, or at most of a limited number, so that here we are concerned chiefly with the growth requirements of individual species of organisms in culture media *in vitro*. Accordingly, to obtain pure cultures is an essential requisite of bacteriological technique. Since bacteria are practically omnipresent, it is necessary to destroy all extraneous organisms which may be in the food media, in the vessels containing these media, and on all instruments which are to come in contact with the cultures. The technique of this destructive process is called sterilisation. Further, the growth of bacteria in other than their natural surroundings involves the preparation of sterile artificial food media; when such media have been prepared we must consider the technique of the separation of micro-organisms from mixtures of different species, and the maintaining of pure cultures when the latter have been obtained. Originally the general principle observed in the artificial culture of bacteria was that the medium used should resemble that on which they grow naturally. In the case of pathogenic bacteria the medium therefore should resemble the fluids of the body. Thus blood serum is often used. Other media have been found which can support the life of most of the pathogenic bacteria. These consist of proteins or carbohydrates in a fluid, semi-solid, or solid form. It is an advantage to have a variety of media, since

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bacteria. A great ad-

vantage resulted when Koch, by adding gelatin to broth, provided a transparent

solid medium in which growth characteristics of particular bacteria become evident. Many organisms, however, grow best at a temperature at which this nutrient gelatin is fluid, and therefore another gelatinous substance of carbohydrate nature called agar, which does not melt at a temperature much below 100° C., was substituted. The reaction of the medium is important, since most bacteria, in contrast to moulds, require a neutral or slightly alkaline reaction. While such media have been of great service in furthering the study of bacteriology, they can afford only a limited insight into the growth requirements of organisms. Accordingly these have been investigated as a metabolic problem. Much information has been gained by starting with a relatively simple 'synthetic medium' containing chemical ingredients of known constitution and ascertaining whether it supports growth or, if not, what other substances must be added in order to obtain cultures.

The chemical components required for growth are those elements which make up protoplasm, and also substances which supply sources of energy. Organisms can be graded according to the complexity of the substances which satisfy their requirements in these respects. The great majority of bacteria grow and obtain energy in the dark, *i.e.* they are chemosynthesising, not photosynthesising. In fact, light is harmful to them. Knowledge is extremely deficient as to the means whereby the new material of bacterial cells is constructed. As regards carbon and nitrogen, great differences exist in the materials from which different species of bacteria can supply their needs. Certain bacteria can assimilate carbon dioxide while utilising nitrogen, etc., from inorganic sources; these are 'autotrophic'. Some of them are obligate autotrophs, *e.g.* *Nitrosomonas*, *Nitrobacter*, and the bacteria which oxidise sulphur or its inorganic compounds. Others, facultative autotrophs, can also utilise organic compounds. Thus the autotrophic bacteria resemble the higher plants in their capacity to utilise inorganic carbon and nitrogen. As a rule bacteria require their sources of carbon to be in a more reduced form than carbon dioxide—such organisms are called 'heterotrophic', the pathogens are included in this group. As regards sources of nitrogen, some heterotrophic organisms can assimilate nitrogen from simple sources, while others are restricted more or less to certain amino-acids as specific components; also particular accessory growth-promoting substances may be required. The nitrogen of the air is assimilated by certain soil bacteria and others use nitrates. Many heterotrophic organisms, however, can use the nitrogen of ammonia salts to synthesise essential amino-acids, particularly tryptophane, provided a suitable organic source of carbon is present; these may be termed 'non-exacting' as regards their nitrogen supply. Others are 'exacting' and can only utilise more complex sources of nitrogen such as amino-acids. Even in a given species, *e.g.* the typhoid bacillus, while the majority of strains are exacting, certain ones are non-exacting and can utilise ammonia. The degradation products of protein, especially amino-acids, are very widely utilised, also the amides of

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In general a

suitable medium for many organisms, including the pathogenic varieties. Complete proteins as a rule do not appear to be utilised as food-stuffs. Certain organisms may form products, *e.g.* indole, produced by *B. coli* and other related species fail to do so. The presence of a fermentable sugar prevents the formation of indole.

While carbon is also provided by the more complex nitrogenous compounds, carbohydrates are in addition extensively utilised as food-stuffs, these are fermented, and this process probably serves the double purpose of liberating energy and of providing the most suitable types of carbonaceous compounds for assimilation. Salts of organic acids, e.g. citrates, lactates, etc., may serve as a source of carbon to some species. According to Quastel, pyruvic acid is the form in which carbon is assimilated by *B. coli*, and a carbonaceous substance to be utilised must be convertible by the bacterial enzymes into this compound. The tubercle bacillus finds in glycerol a particularly suitable source of carbon.

The capacity of certain species of bacteria to ferment particular sugars, etc., constitutes an important biological property which is utilised for their classification and identification. The breakdown of the fermentable substance with the production of acid products and also frequently gases (carbon dioxide, hydrogen, etc.) is determined. Besides sugars, allied bodies such as alcohols, glycosides, and other compounds may be acted on. Since a mixture of several species of bacteria (or these species acting in sequence) may exhibit 'synergic action', gas being produced from a substance which does not yield gas when acted on by each organism alone, it is essential to ensure that the cultures under examination are pure.

By 'adaptation' or 'training' bacteria may acquire the power of assimilating or breaking down substances either nitrogenous or carbonaceous which they did not originally act upon. For instance, an exacting strain of the typhoid bacillus can be induced to grow in a medium lacking tryptophane and to synthesise this compound. Such changes, in some cases at least, seem to depend on the organisms acquiring the power to form 'adaptive' enzymes (*vide* p. 22) which possess an affinity for the substrates in question. Strains of *B. paratyphosus B* and the typhoid bacillus, which are non-exacting as regards nitrogen-source etc., in the presence of oxygen, are more exacting under anaerobic conditions.

While a large number of organisms will grow indefinitely on synthetic media of known composition, there are some which cannot be cultivated under these conditions, e.g. *Streptococcus pyogenes*, pneumococcus, and Pfeiffer's bacillus. It would appear that in the case of these, accessory food factors are required. These accessory principles, as shown by Knight and Fildes, are vitamin-like in the sense that they are effective in promoting growth when present in minute amounts, so that they do not contribute significantly to the nitrogen or carbon required to make up the new-formed protein. They can be derived from various sources, e.g. extract of yeast, urine, etc. It is important that organisms which are capable of flourishing on synthetic media, themselves produce the growth-promoting substances. Pfeiffer's bacillus requires two accessory factors, one of which is supplied by
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nicotinic acid and aneurin (vitamin B₃) along with amino-acids and glucose, under anaerobic conditions pyruvic acid and uracil, as vitamin, are needed in addition. Some strains of the diphtheria bacillus require several growth factors, one of which is pimelic acid.

A comparison of the nutrient requirements of closely related organisms, some of which are commensals of relatively low pathogenicity while others

are typical pathogens, such as those of the coli-typhoid group, has indicated that the more specifically pathogenic members have the more complex nutritive requirements. Thus *B. coli* can grow with ammonia as the sole source of nitrogen; and it synthesises certain amino-acids which are indispensable for building up its protoplasm—'essential metabolites'. The typhoid bacillus cannot synthesise these amino-acids from ammonia; but as it is also unable to grow without them, they must be added ready-made to the culture medium, i.e. they are, for this organism, 'growth factors', in other words essential metabolites which it cannot synthesise. Similarly, glutamine is a growth factor for most recently isolated pathogenic streptococci, but after prolonged cultivation *in vitro* the addition of this factor is not necessary (Fildes *et al*)

Certain highly parasitic bacteria, e.g. gonococcus, meningococcus, etc., fail to grow in the absence of blood serum, and the growth of many pathogenic species is promoted in the presence of serum. The bacillus of Johne's disease exhibits a peculiar requirement, viz. some substance synthesised by other acid-fast bacteria; thus dead tubercle bacilli or timothy grass bacilli added to the medium enable it to flourish. Organisms which when recently isolated are highly exacting *in vitro* often grow much more readily after repeated subculturing.

As regards *inorganic constituents*, potassium or sodium and phosphorus, chlorine, sulphur, and carbon—in the form of phosphates, chlorides, sulphates, and carbonates—would appear to be the only essentials for many organisms, while others may require also magnesium, calcium, or iron; but it is difficult to say how far minute traces of other minerals are of importance. Certain autotrophic sulphur bacteria (*Beggiatoa* and *Thiothrix*) assimilate sulphuretted hydrogen and store in their bodies granules of sulphur, which disappear when the organisms are starved.

It should be noted in regard to culture media generally, that filtration through paper, cotton wool, etc., may by adsorption remove essential accessory food substances. Most bacteria seem to form products which are unfavourable to their own vitality, for when a species is sown on a mass of nutrient medium it soon ceases to grow, even before the food supply is exhausted, alteration in hydrogen-ion concentration explains only in part the cessation of growth. Also, substances such as many of the amino-acids, when present in excess, tend to inhibit growth. When the food supply of an organism fails, it degenerates and dies. The proof of death lies in the fact that when it is transferred to fresh suitable media it does not multiply. If the organism forms spores it may survive the want of food for a very long time. A distinction must be drawn between the conditions in media which promote the maximal growth of organisms and those which lead to the longest persistence of vitality.

Moisture. The presence of water is necessary for the continued growth of all bacteria. The amount of drying which bacteria in the vegetative stage will resist varies very much in different species. Thus at room temperature the cholera vibrio is killed by two or three hours' drying, while the *Staph. aureus* will survive ten days' drying, and the diphtheria bacillus still more. In the case of spores the periods are much longer. Spores of the anthrax bacillus will survive drying for many years if kept in the dark. While the above statements apply to drying as ordinarily carried out, intense rapid drying and preservation *in vacuo* maintain the vitality of many vegetative forms for long periods.

Respiration and Relation to Gaseous Environment. The respiratory mechanisms of bacteria have attracted much attention, particularly in

relation to the processes of cellular oxidation and reduction. Observations have demonstrated the complexity of the mechanisms involved; and it must be noted that bacteria vary widely in their oxidative processes, and even with any one species, variations may occur according to the conditions of life and growth. The term respiration is now applied generally to the chemical processes whereby the living organisms liberate energy from substances either in the presence or absence of oxygen, and it is the occurrence of such processes within the living cells which is of use for their nutrition and growth. The original conception of respiration implied the addition of oxygen to the substance acted on; but oxidation has come to include also reactions in which hydrogen is removed (dehydrogenation) or electrons are lost (as in the transformation from the ferrous to the ferric state). It is necessary to bear in mind that a proportion of the energy set free in such reactions may not be convertible into work under the given conditions.

Oxygen. The relation of the oxygen of the air to bacteria is such an important factor in their life that it enables a broad biological division to be made among them. Some bacteria live and grow best when there is free access of oxygen from the air. To these the title of *aerobes* is given. Other bacteria will only grow when free oxygen is absent, they require also a medium which possesses a suitable degree of reducing action. These are called *anaerobes*, but different species show a wide range in their intolerance of oxygen. In still other bacteria the amount of oxygen is a matter of indifference within wide limits; such organisms are usually denominated *facultative anaerobes*—being aerobic but capable of growing with very little oxygen. Examples of aerobes are *B. tuberculosis* and *B. subtilis*, of a strict anaerobe, *B. tetani*, while the great majority of pathogenic bacteria are facultative anaerobes. Those bacteria which in a deep culture show most abundant growth at a point a short distance below the surface, have been designated *micro-aerophilic* on the view that they flourish best in the presence of a minute trace of oxygen; but this effect is often an indication of their carbon dioxide requirement.

The luminescent bacteria isolated from sea water are a striking example of the necessity in certain cases of molecular oxygen for function, in the absence of the gas the cultures cease to give out light. A marked feature of bacterial oxidation is that substances may be attacked which otherwise are very stable at ordinary temperatures. When substances are rendered liable to chemical change in this way they are said to be 'activated'. Enzymes produced by the organisms are responsible for such activation. Aerobic oxidations will be considered first. The transfer of hydrogen to molecular oxygen is shown by placing a washed suspension of the organisms from a culture ('resting bacteria') in a manometric apparatus along with a substance which they oxidise. It is then found that oxygen is absorbed. Living *B. coli* acting on lactose produce this one. This is shown by the fact that occur if the organisms are first of fluoride, the reaction is restored by the addition of a trace of certain substances such as methylene blue. The latter, owing to its capacity for being reversibly reduced and oxidised, acts as a hydrogen 'carrier'. Such carriers play an important part in bacterial oxidations; examples are the so-called respiratory pigments—the cytochrome system, flavoprotein—and glutathione. placing the subst such as glycerol, a lower fatty acid, an amino acid, resting bacteria along with some substance which is susceptible to reduction (a

hydrogen acceptor). Methylene blue is much used for this purpose because its reduction to the colourless leuco compound provides an indicator of the reaction. Many bacteria, especially those which are pronounced aerobes, possess *cytochrome*, which can be demonstrated spectroscopically. The reduced form is present in suspensions which have stood for some time, or is formed quickly on passing nitrogen gas through the liquid; the oxidised form is produced through the mediation of cytochrome oxidase when the suspension is shaken with air. The anaerobic bacteria are devoid of cytochrome, as also are some streptococci. Another respiratory pigment, *flavoprotein*, is demonstrable by the spectroscope in various bacteria and seems to be especially important in anaerobes. Some organisms give a positive nitroprusside reaction suggestive of *glutathione*. Oxidations by molecular oxygen are as a rule effected in a series of stages, for some of which enzymes are responsible. It has been shown in a number of cases that substances are incompletely oxidised by intact bacteria, but undergo complete oxidation when the organisms have been poisoned in certain ways. This difference probably affords a measure of the assimilative activity of the normal cells. Peroxide of hydrogen is formed in the course of many bacterial oxidations, and in some cases, where peroxidase is also produced, may serve to oxidise certain substances for the use of the bacterial cells. When the organisms are sensitive to peroxide, accumulation of this compound will lead to their death. But this is often prevented by their forming at the same time catalase which decomposes the peroxide. The *pneumococcus* forms peroxide, but not catalase, and is sensitive to the former, so it is rapidly killed in this way in ordinary media.

Anaerobic respiration Facultative anaerobes when growing in the absence of molecular oxygen can only obtain energy for building their cells by oxidising certain constituents of the medium at the expense of others, which become reduced at the same time. This process of oxido-reduction yields less energy than when oxygen is the hydrogen acceptor. Occasionally one molecule of a compound is oxidised at the same time that another of the same kind is reduced—dismutation, but as a rule two different substances are involved. Thus the requirements for anaerobic growth are more complex than for aerobic, e.g. various substances (lactate, succinate, acetate, glycerol) act each as the sole source of carbon for *B. coli* growing under aerobic conditions, but none of these suffices by itself anaerobically, although in the presence of nitrate they do so owing to the latter acting as a hydrogen acceptor under the influence of nitratase which the culture contains. Sugars are the most important sources of energy and carbon for fermenting organisms under anaerobic conditions. The bacteria which are obligate anaerobes belong to a unique class of living agents discovered by Pasteur. Their intolerance of molecular oxygen is such that in the case of the most sensitive members minute traces prevent growth. They will grow in a closed space if the oxygen is removed, e.g. by displacement with an indifferent gas such as hydrogen or nitrogen, or by absorption, say with an alkaline solution of pyrogalllic acid, or along with a culture of an aerobe, owing to the latter absorbing the oxygen of the contained air. Growth in liquid medium exposed to the air occurs provided that substances are present which produce a sufficient reducing tendency, such as ascorbic acid, cysteine, thioglycollate, metallic iron, or particles of minced muscle. The obligate anaerobes possess special mechanisms for dehydrogenation, thus *B. sporogenes* attacks certain amino-acids, but does not affect substances acted on by *B. coli*. Also, the chief hydrogen acceptors in the case of facultative anaerobes do not act as such for *B. sporogenes*, while some amino-acids have this property.

The nature of the reaction between free oxygen and the obligate anaerobes is not clear. McLeod and Gordon ascribed the anaerobic character of organisms, such as *B. tetani* or *B. sporogenes*, to their forming peroxide of hydrogen in cultures in the presence of oxygen; at the same time they fail to form catalase, and as they are extremely susceptible to peroxide, growth cannot go on in the presence of air. The evidence adduced for peroxide formation (green coloration in haemoglobin-containing medium) is not conclusive, however. In the case of *B. tetani*, etc., the harmful effect of oxygen is intensified by the presence in the medium of traces of certain copper compounds (O'Meara)

Oxidation-reduction potentials. When bacteria are growing in a culture medium such as broth, it is usually found that there is a reducing tendency in the system which is greater than that of the broth itself. This has long been known from the fact that reducible substances, such as dyes, when introduced into fluid cultures are often converted into the colourless leuco products (the original colour being restored by vigorous oxygenation). At the same time, as we have seen, oxidation processes are going on by which

materials required for life and proliferation
culture re-
which give

rise to changes in electrical potential. The intensity of these changes can be measured by an apparatus illustrated in Fig 2. For details reference should be made to special works. In principle an unalterable electrode, consisting e.g. of platinum foil, dips into the fluid culture medium in the vessel *a*; this constitutes a 'half cell'. Also dipping into the culture medium is one end of a U-tube *b* filled with agar made up with a saturated solution of KCl. The other end dips into the vessel *c* also containing saturated KCl solution; *c* communicates by a tube containing the same solution with a 'standard half cell' *d* consisting of a layer of calomel as electrode superimposed on mercury and covered by a saturated solution of KCl. Wires leading from the platinum electrode and the mercury of the standard half cell are connected to a potentiometer, calibrated to ± 1 volt and reading to the nearest millivolt, and a galvanometer. In this way, the potential of the standard half cell being known relative to that of the hydrogen electrode, the potential of the system can be measured in volts. This is the electrode potential, E_h , it is negative when the system is a reducing one and positive when it is oxidising. The pH must be kept constant, as variations affect the electrode potentials. Another method of estimating E_h depends on the fact that a number of dyes, including indophenols, toluylene blue, thionin, methylene blue, indigo sulphonates, safranins, neutral red, etc., change colour on reduction over different E_h ranges and so can be used as indicators. (These are given in order over a wide range from positive to negative.) Their use is attended by various difficulties, however, since they may themselves participate in the oxidation-reduction reactions. Also they may exert catalytic or toxic effects. Further, their colour frequently depends on the pH as well as the E_h . According to Knight and Fildes the spores of *B. tetani* cannot germinate if the E_h is higher than about +0.1 volt, and for growth of the strict anaerobes the E_h must have a value between -0.006 and -0.436 volt.

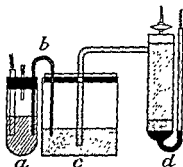


FIG. 2. Arrangement of cell for measuring oxidation-reduction potentials.

By following the behaviour of Eh in cultures at different stages of growth under the usual conditions, Hewitt has found that the reducing state varies according to the particular organism. During the logarithmic phase of growth the aerobes in general produce a moderately intense reducing level, but many are unable to effect further reductions. The anaerobes can only effect reduction when the medium is partially reduced to begin with, e.g. by exclusion of the oxygen of the air, they are then able to produce and maintain intense reducing conditions. Therefore the distinguishing feature between aerobes and anaerobes is not the potential ultimately reached in the culture, but the potential range at which they are able to start growing. With some organisms, such as the pneumococcus, the level of reducing intensity is not maintained after the logarithmic phase of growth, highly oxidising conditions becoming gradually established; this is associated with the formation of peroxide.

Other gases With regard to anaerobes, hydrogen and nitrogen are indifferent gases. Many anaerobes, however, do not flourish well in an atmosphere of carbon dioxide. *B. abortus*, on the other hand, when freshly recovered from the tissues, requires for its growth the presence of a considerable excess of carbon dioxide. Also, according to the observations of Gladstone, Fildes and Richardson, the presence of carbon dioxide is essential for the growth of many pathogenic organisms. The formation of toxin and hæmolysin by staphylococci is increased in an atmosphere rich in carbon dioxide. The mode of action of this gas in favouring bacterial growth is uncertain. It may be directly assimilated or participate in oxido-reduction or enzyme reactions, also in some cases it may prevent excessive accumulation of alkali. Recently it has been shown that heavy carbon (C_{13}), supplied as carbonate, appears in the carboxyl group of succinic acid produced by growing *B. coli* in the medium (Wood *et al.*). Few experiments have been made on the action on bacteria of gases under pressure. Increased pressures of carbon dioxide and of oxygen both prevent the proliferation of *B. pyocyaneus*, but whereas the former soon proves lethal, very high pressures of oxygen are merely inhibitory, the culture to normal atmospheric pressure. *B. pyocyaneus* is killed by exposure either to oxygen or carbon dioxide at pressures, while many coliform bacilli will survive high pressures of both. Hydrogen at high pressures is not harmful.

Temperature. For every species of bacteria there is a temperature at which it grows best, this is called the 'optimum temperature'. There is also in each case a maximum temperature above which there is no growth, and a minimum temperature below which growth does not take place. As a general rule the optimum temperature is about the temperature of the natural habitat of the organism. It must be noted, however, that the optimum temperature for multiplication need not be the optimum for other vital functions. For organisms taking part in the ordinary processes of putrefaction the temperature of warm summer weather (20° to 24° C.) may be taken as the average optimum, while for organisms normally inhabiting animal tissues 35° to 39° C. is a fair average. The lowest limit of ordinary growth is from 12° to 14° C., and the upper is from 42° to 44° C. In exceptional cases growth may take place as low as 0° C., and as high as 75° C. Some organisms which grow best at a temperature of from 60° to 70° C. have been isolated from animal manure, the intestinal tract, etc., these have been called 'thermophilic'. Some organisms grow above a certain limit, some are killed by heating beyond a certain limit, and some are killed by heating beyond a certain limit.

merely paralysed. Especially is this true of the effect of cold on bacteria. The results of different observers vary, but if we take as an example the cholera vibrio, Koch found that while the minimum temperature of growth was $6^{\circ}\text{C}.$, a culture might be cooled to $-32^{\circ}\text{C}.$ without being killed. When kept immersed in liquid air (about $-190^{\circ}\text{C}.$), the typhoid bacillus and *Staph aureus* were still living after six months (Macfadyen). With regard to the upper limit, harmful effects are usually observed a few degrees above the optimum, and ordinary organisms in a spore-free condition will seldom long survive a temperature of $55^{\circ}\text{C}.$ This appears to be related to the temperature at which the respiratory enzymes are destroyed. Many organisms lose some of their properties when grown at unnatural temperatures. Thus many pathogenic organisms lose their virulence if grown above their optimum temperature, and at $42^{\circ}\text{C}.$ the anthrax bacillus may also lose permanently the faculty of forming spores. Some chromogenic forms, most of which prefer rather low temperatures, may fail to produce pigment at higher tem

in 2 }
 represents the extreme range for most pathogenic organisms; beyond these limits they are inhibited or killed. In certain cases there is special susceptibility or tolerance towards either the acid or the alkaline side. Thus the cholera vibrio is very sensitive to acid and fails to grow in peptone water at a pH under 5.8, on the other hand, it flourishes at a pH of 8 and tolerates a pH up to 10.6. The lactobacilli are 'aciduric' organisms (i.e. tolerate acid, sometimes called 'acidophilic') and in culture can develop a pH of under 3.4. Enterococci have a very wide range for growth, from pH 5 to pH 11.

Light. The purple and brown bacteria and purple and green sulphur bacteria are the only members of the bacterial class in which assimilation is aided by exposure to light, either in the visible range or infra-red, this is due to their pigment, which contains a constituent closely related to chlorophyll. All other bacteria are damaged in their vitality by light rays. Bacterial pigmentation, however, is often intensified when the cultures are brought into the light.

The Methods of Bacterial Action: Enzymes. The chemical actions of bacteria depend on the production by them of ferments or enzymes, biological catalysts of a very varied nature and complicated action. Thus the liquefaction of gelatin or coagulated serum is due to proteolytic enzymes which pass into the medium in which organisms are growing. Ferments which invert sugar, which split up sugars into alcohols or acids, which coagulate casein, which form ammonium carbonate from urea, also occur. The enzyme hyaluronidase (spreading factor), which is produced by some infective organisms, increases the permeability of tissues and so may be a factor influencing the extent of invasion (Duran-Reynals).

Enzymes are apparently all of protein nature. They may diffuse readily into the surrounding fluid, as in the case of gelatin-liquefying organisms. Frequently the enzymes pass into solution as a result of autolysis of the organisms. Again, they may be retained in the cells where they are formed, in the latter case the bacterial protoplasm often must be thoroughly disintegrated, e.g. by grinding, before the ferment is liberated, as in the original preparation of zymase from yeast by Buchner. Cell-free bacterial enzymes acting on carbohydrates have seldom been got, e.g. the lactic dehydrogenase of *B. coli*, prepared by autolysing the washed bacteria along with 1 per cent potassium fluoride and filtering (Stephenson). That a purely intracellular digestion may take place is illustrated by what has been shown to occur in

the case of the *Micrococcus ureæ*, which forms ammonium carbonate from urea. Here, if after the action has commenced the bacteria are filtered off, no further production of ammonium carbonate takes place, which shows that no ferment has been dissolved out into the urine. If now the bodies of the bacteria be extracted with absolute alcohol or ether, either of which of course destroys their vitality, a substance is obtained of the nature of a ferment, which, when added to sterile urine, rapidly causes the production of ammonium carbonate. This ferment has evidently been contained within the bacterial cells. The multiplicity of the enzymes formed by different organisms and even by the same organism presents a complicated problem.

Adaptive and constitutive enzymes. In the investigation of the phenomena of the ferment action of bacteria, it has been noted in certain cases that the enzymes formed depend on the substrate offered to the organisms. Thus a suspension of *B. coli* which had been grown on agar and contained no enzyme acting on formate, was brought into contact with formate in broth; after half to one hour hydrogen was evolved, although no multiplication of the organisms occurred up to three hours. Here selection is excluded. It is noteworthy, however, that only living organisms exhibit such production of enzymes. Other adaptive enzymes are developed slowly, as when the typhoid bacillus growing in fluid medium containing dulcitol at first causes no change in reaction, but later produces acid. Then after repeated subcultivation in the presence of dulcitol a strain evolves which produces acid quickly. The acquired property may be retained on further cultivation in medium lacking the substrate. Constitutive enzymes, on the other hand, are formed irrespective of the nature of the medium.

Autolysis. res and at the same time undergo autolysis. ical changes (involution forms, vide p. and, if originally Gram-positive, cease to retain the stain, many they disintegrate and dissolve. These changes appear to be the result of enzymes contained in the organisms. The meningococcus, gonococcus, and pneumococcus are striking examples, but the process occurs with many organisms. The meningococcus contains lysins which act both on itself and on various other organisms; exposure to a temperature of over 70° C. for thirty minutes is required for their inactivation (Flexner). With the pneumococcus it has been found that the autolytic enzymes can be obtained in solution. They are inactive towards most other organisms, but act on the closely related *Strept. viridans*. Low concentrations of iodine, formaldehyde, acetic acid, etc., inhibit the autolysis, but when these reagents are removed the process sets in; larger amounts destroy the autolysin. Sodium desoxycholate and other surface-active agents hasten autolysis, so that when added to a fluid culture they cause it to clear almost at once. Treatment by cold acetone followed by suspension in saline, also freezing and thawing have a similar effect. By the use of a small amount of the enzymes autolysis is modified, so that the pneumococcus becomes Gram-negative without undergoing further structural changes.

The Parts played by Bacteria in Nature. The chief effect of bacterial action in nature is to break up into more simple combinations the complex molecules of the organic substances which form the bodies of plants and animals or which are derived from them. The changes which organic substances undergo in being split up by bacteria depend, first, on the chemical nature of the substances involved, and, secondly, on the varieties of the bacteria which are acting. For an exact knowledge of the capacities of any particular organism there must be an accurate chemical examination of its effects when it has been grown in artificial media the nature of which is known. That the

very complicated process of putrefaction is due to bacteria was first proved by Pasteur, since any organic substance can be preserved indefinitely from ordinary putrefaction by the adoption of some method of killing all bacteria present in it, as will be afterwards described. The destruction of protein substances which is mostly involved in the wide and varied process of putrefaction, can be undertaken by different varieties of bacteria. The action of the latter is analogous to what takes place when proteins are subjected to gastric and intestinal digestion. In these circumstances, therefore, the production of albumoses, peptones, etc., similar to those of ordinary digestion, can be recognised in putrefying solutions, though the decomposition always goes further, and there results a mixture of simpler substances, including fatty acids and bases, ammonia, carbonic acid, methane, sulphuretted hydrogen and other foul-smelling gases, and indole. The processes as observed under natural conditions, are exceedingly complicated, and different bacteria (especially anaerobes) are concerned in the different stages. An indication of slight proteolytic action is afforded by liquefaction of gelatin, fibrin, or coagulated serum or egg-white, such properties are exhibited by a number of pathogenic organisms.

Bacteria, as well as yeasts and higher fungi, are responsible also for the analogous processes of breaking down of carbohydrates which are comprised in the term fermentation. The formation of particular substances in marked amounts by certain species, or even strains, of bacteria finds a valuable economic application, e.g. the production of acetic, butyric, propionic, and lactic acids, various alcohols (ethyl, butyl, etc.), acetone and methane, and other gases, etc. Such processes may be wasteful, as in the spoilage of milk and the development of rancidity in butter, or they may be economically important, e.g. in the ripening of cheese or the manufacture of vinegar and acetone. A common result of bacterial action is an alteration of the pH of the medium, sometimes towards the acid, sometimes towards the alkaline side. A reversal of reaction may occur, e.g. acid substances being produced at first, and these then being broken down further with the formation of less acid products.

The disintegration of organic material, which is so prominent an effect of bacterial growth, is, of course, an accompaniment of the synthesis of the complex substances of which the bacteria themselves are built up. The most striking example of such synthetic power is presented in the case of those bacteria which in the soil make nitrogen available for plant nutrition by converting ammonia into nitrites and nitrates. Winogradsky, by using media containing non-nitrogenous salts of magnesium, potassium, and ammonium, and free of organic matter, demonstrated the existence of forms which convert, by oxidation, ammonia into nitrites, and of other forms which convert these nitrites into nitrates. Both can derive their necessary carbon from alkaline carbonates. Other bacteria can actually take up and combine into new compounds the free nitrogen of the air. Such is *B. radicicola* (*Rhizobium leguminosarum*) found in the tubercles which develop on the rootlets of the leguminosae. Without such organisms the tubercles do not develop, and without the development of the tubercles the plants are poor and stunted. Bacteria thus play an important part in the enrichment and fertilisation of the soil. On the other hand bacteria are also found in the soil which reduce nitrates to nitrites and nitrites to ammonia. The herbivora, on which the nutrition of carnivora depends, are enabled to utilise vegetable matter as food in virtue of the cellulose-splitting bacteria which flourish in their alimentary tract.

A certain comparatively small number of bacteria have been proved to

other properties. Thus it may agglutinate spontaneously in 0.85 per cent. NaCl solution, although forming a stable suspension in weaker concentrations. A very important character in which the two types may show differences concerns their immunological properties due to alterations in the antigens. Here it may be said that serologically the two forms may be distinct—an antiserum to the S form agglutinates not only the homologous smooth culture, but also all smooth cultures of the organism; on the other hand, the S antiserum has little action on any of the R variants, whereas these are agglutinated by an anti-R serum, which contains no agglutinins for the S form. Similarly S and R forms have been obtained from many other organisms, including the coli-typhoid group, the *Pasteurella* group, the anthrax bacillus, and also the streptococci and pneumococcus. There are frequently differences also in microscopic appearances of the individual organisms constituting the two types of colony, thus, in the case of capsulate organisms, e.g. the pneumococcus, the rough variant may be devoid of a capsule. The different types of colony may correspond with marked differences in virulence, as De Kruif found in the case of a *Pasteurella* organism. Here the S type was highly virulent and the R type non-virulent. Similar observations have been made for other organisms, e.g. the typhoid bacillus and the *Salmonella* group, the 'normal' forms of which are smooth. But the rough form is not necessarily the one which lacks virulence, e.g. it is the smooth form of the anthrax bacillus which is non-virulent. Other forms have also been described, e.g. in the case of group A streptococci, 'mucoid' and 'matt', among which are included the virulent cultures. Arkwright showed for *B. paratyphosus* A that vaccines of the R type, which was the less virulent, did not confer protection against infection with the S type. Once organisms have passed into the non-virulent R condition, it appears that they are not readily susceptible to conversion again into the S type by procedures such as frequent subculture or animal passage. In order to obtain variants which are persistent in character, repeated plating and selection of colonies for subculture may be required.

In the *Salmonella* group again, variants exist which are characterised solely by the possession of 'group' and 'specific' antigens respectively; these differences are not permanent, and they are not associated with any distinctions in appearance of colonies. A variation of a very striking kind which is of permanent nature, has been induced experimentally in pneumococci. A suitable living culture which has lost the power to form capsules and so has become non-virulent, can be changed into the capsulate virulent form of another type by allowing growth to occur *in vitro* in a medium containing a minute concentration of sterile substances derived from a capsulate culture of the latter type. The evidence points to the transforming substance being a desoxyribonucleic acid.

As has been mentioned already in connection with adaptive enzymes variations in the biochemical or fermentative actions of bacteria are often met with, these have been especially studied in the coli-typhoid group, as will be afterwards described. Thus new chemical characters may be acquired, and this may often occur spontaneously in culture, but is met with especially under two conditions, namely (a) a gain in fermentative properties when the organisms are grown for a long time on a medium containing a particular constituent, e.g. a sugar, or (b) a loss of such properties when a substance somewhat inimical to growth is added to the medium—in both cases the organisms undergo adaptation. The occurrence of such variations is sometimes indicated by the appearance of papillæ on certain of the colonies on a solid medium. When the papillæ consist of organisms which have acquired

the power of fermenting a substance present in the medium and an indicator such as neutral red is also added the papillæ are of different colour—red on white colonies. In many of the examples given the variants revert to the original type after subculture, but in certain instances they are permanent. The latter occurrence was observed first by Neisser and Massini in the case of '*Bact. coli mutabile*', and similar phenomena were afterwards observed and fully described by Penfold. Later work of Lewis has shown that in this case the capacity to ferment lactose is a variation which occurs in a small proportion of individuals in the culture quite independently of the presence of lactose. Twort, by growing the typhoid bacillus for a long time in lactose medium, found that it acquired the property of fermenting this sugar, but this is a rare result and others have failed to effect the change. The evidence points to such variants being the result of adaptation rather than mere selection.

As regards the significance of microbic variation, the changes brought the life his view a patho-
genic bacteria at least, the association of alterations in colony form with with a phenom-
ist and parasite
non-virulent R
protective mechanism in the host.
when they are derived from single
hanism. Although no sexual

processes have been demonstrated in bacteria, the existence of multiple nuclear structures in a single organism, which has been claimed, would allow such events.

In general, it may be said that the variations described among the pathogenic bacteria in no way interfere with the accepted biological classification. The species recognised retain certain basic characteristics, any variations observed represent subsidiary differences from the standard type. The subject of variation will be considered further in relation to virulence and to individual bacterial species, and will also be dealt with in its immunological aspects.

THE EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON BACTERIA—STERILISATION, ANTISEPTIC ACTION

Conditions favouring the multiplication and other vital activities of bacteria have been discussed. It may be said that all other conditions are inimical, and in this connection it should be noted that many species of organisms fail to survive long in distilled water, e.g. the cholera vibrio. But special attention is paid to those agencies which are either rapidly lethal or powerfully inhibitory. Since bacteria are ubiquitous in the neighbourhood of higher forms of life, it is necessary to exclude extraneous organisms preparatory to the study of the relations of bacteria to disease and other processes. Therefore the killing of organisms (*sterilisation*) is essential in the preparation of all culture media and for receptacles intended to contain materials for bacteriological examination. For all the above purposes the bactericidal agency should not persist after its action has been completed. Further, infected articles must be rendered harmless as rapidly as possible by processes of disinfection. The death of bacteria is judged by the fact that when they are placed on a suitable food medium multiplication does not

take place. Although not killed, bacteria may undergo other changes as the result of physical or chemical agencies. Thus their capacity to proliferate may be reduced or they may alter in their morphology, developing filamentous forms and appearances like those due to autolysis; also their metabolic processes may be reduced or altered and virulence diminished.

The antibacterial action of serum and the treatment of infective diseases by administering agents which conduce to the destruction of the causal organisms or inactivation of their products, will be dealt with separately under Immunity, Serotherapy, and Chemotherapy; bacteriophage is also considered by itself.

PHYSICAL AGENCIES

Moist heat This is one of the most effective and easily applied bactericidal agents. In watery medium the majority of non-sporing organisms are killed within a half to one hour at 55° to 60° C., although higher temperatures may be required, e.g. 70° C. for some staphylococci. The heating of food-stuffs, such as milk, in this way to increase their keeping qualities is called 'pasteurising'. Members of the coli-typhoid group after heating at 58° C. may fail to multiply on culture media when exposed to the oxygen of the air and yet grow anaerobically (Oerskov). The term 'thermal death point' is sometimes applied to the temperature required to sterilise a bacterial suspension in a standard time, while 'thermal death time' is the time required to effect sterilisation at a given temperature; however, both are somewhat indefinite, since the resistance of the same species of organisms tends to vary. Increase in temperature has a very great effect in shortening the time required for sterilisation. Thus within the range of 10° C. from 49° C. to 59° C. the sterilising time for typhoid bacilli is reduced from 196 minutes to 1.26 minutes (Chick). Raising momentarily to the boil is fatal to vegetative forms of all organisms. Spores are more resistant and withstand boiling for a variable period, which depends on the species, but also, spores of the same species vary widely among themselves in their resistance to heat. While spores of *B. subtilis* may withstand boiling for many hours, those of the common pathogenic sporing organisms, e.g. the anthrax bacillus, are killed in ten minutes. The time required for germination of heated spores which have not been killed, may be greatly lengthened owing to the destruction of some essential products supplied by the growing organisms, but in other cases heating short of a lethal temperature causes accelerated germination. In order to avoid prolonged heating, which may damage culture media, Tyndall's method of intermittent sterilisation is used—heating in a 'Koch's steam steriliser' at 100° C. for twenty minutes on each of three successive days. In this way vegetative organisms are killed on the first exposure; and any spores which survive will have passed into the vegetative state before the second or third heating. *Moist heat at temperatures above 100° C., i.e. saturated steam under pressure*, has a very powerful sterilising action. At a pressure of thirty pounds per square inch, equivalent to 121.5° C., all organisms and spores are killed in a quarter to half an hour. The boiler used for this purpose is called an autoclave. An important property of moist steam is its penetrating power, dry steam is not a more effective sterilising agent than any other hot gas. *Dry heat* is much less effective than moist and also less penetrating. Its applications are limited, e.g. the sterilisation of bacteriological wires by flaming to a dull red heat, and of glass articles plugged with cotton-wool stoppers or wrapped in paper, for which exposure at 170° C. in a 'hot-air oven' for one hour suffices. The action of heat, short of carbonising, is in denaturing the bacterial proteins.

Cold is harmful to certain organisms, *e.g.* the gonococcus and meningococcus die more quickly at 2° to 4° C or room temperature than at 37° C, but most bacteria survive longer at 2° to 4° C than at 37° C. Aqueous suspensions of vegetative bacteria when kept frozen die more quickly at temperatures just below 0° C than at -20° C, due to denaturation of their proteins. *B. pyocyaneus* is particularly susceptible, while spores are unaffected (Haines). Bacteria resist very low temperatures, such as that of liquid air or liquid hydrogen, for long periods.

Drying in the ordinary way quickly kills some organisms, such as the gonococcus, meningococcus, and cholera vibrio, while others, like the diphtheria bacillus and the tubercle bacillus can survive and remain virulent for weeks in the dry state in the dark. Many spores also remain virulent for years when dried on threads or glass beads. Rapid drying from the frozen state ('lyophile process') followed by storage *in vacuo* in the refrigerator is an effective means of keeping organisms alive and virulent, especially when they have been suspended in a medium containing protein, such as dilute serum (Burrows).

Light and other radiations. Light is harmful to all but the photosynthesising bacteria. The most active bactericidal rays are those in the ultraviolet region, which occupy a position in the spectrum at some distance from the visible rays—from 2,960 to nearly 2,100 Angstrom units, as tested with a tungsten arc and quartz spectrograph. The extent varies somewhat in the case of different organisms, but the area of rays in the spectrum effective against any one organism is comparatively sharply marked off. The bactericidal rays have little penetrating power, being completely absorbed by human skin in a thickness of 10 mm and also by glass. Only those rays which are bactericidal to the *Staph. aureus* are absorbed by a suspension of that organism. Toxins also are destroyed and proteins coagulated by ultraviolet radiation. The exact mode of action is not clear. Ultraviolet rays have been applied practically for the disinfection of the air of rooms. Direct sunlight has a very inimical effect on bacteria, as originally discovered by Downes and Blunt. It is chiefly the blue, violet, and ultraviolet rays which are fatal. Exposure of anthrax spores for several hours to sunlight through glass kills them. Typhoid bacilli are also killed, and similar results have been obtained with many other organisms. Under the conditions observed, the capacity of the medium to produce growth of the organisms is not affected. Also the fallacy which might arise from the effect of the heat rays of the sun has been excluded, although light *plus* heat is more fatal than light alone. A powerful electric light is also fatal. Diffuse daylight has an injurious effect on bacteria, but a much longer exposure is required. Another effect of light is to produce peroxide in uninoculated media, which then acts as an antiseptic. The formation of peroxide appears also to account in great part for the *photodynamic action* of solutions of certain dyes, *e.g.* rose bengal, eosin, methylene blue, etc., which when added to a suspension of bacteria rapidly cause their death on exposure to light provided that molecular oxygen is present. The dyes which have this effect are generally those which fluoresce in solution, although there seems to be no close relation between the two effects. The lethal action of α -, β -, γ -rays of radium, X-rays of both hard and soft has been compared by Lea *et al.* the lethal dose being independent of temperature. It varies for different radiations and a paradoxical effect was noted, namely that for *B. coli* the dose was greatest with the radiations which produce ionizations closest together, *i.e.* α -rays, and smallest with β -rays,

with which ionizations are farthest apart, whereas for spores of *B. mesentericus* the lethal effect was graded in the reverse order. It is supposed that death of an organism is due to an ionizing particle passing through a specially sensitive region, a target, which may be a gene.

Vibrations of sonic and supersonic nature have a killing effect on bacteria which may not be purely mechanical. *Mechanical shaking* of a suspension of organisms with solid particles, *e.g.* glass beads of varying sizes, produces death and disintegration of vegetative forms and spores within several hours.

CHEMICAL COMPOUNDS—ANTISEPTICS

From Koch's time onwards, owing to the importance of being able to kill or inhibit bacteria, an enormous amount of investigation has been directed to the means of doing so by chemical agencies. A substance having such a capacity is called an antiseptic, and the term is usually applied to substances which act in relatively high dilutions. Most antiseptics are general protoplasm poisons harmful to all forms of life, higher as well as lower, so that their use is limited to the inhibiting or killing of bacteria outside the animal body; still even this is of high importance. But all powerful antiseptics do not coagulate proteins such as serum or egg albumen. A distinction is often drawn between a *bactericidal* or *germicide* agent, which sterilises organisms rapidly in high dilutions, a *bacteriostatic* agent, which prevents multiplication, although causing death only slowly, and an *antiseptic*, which interferes with the vital activities of bacteria, but does not necessarily kill them. However, the one property tends to merge into the others.

Methods of Estimating Antiseptic Activity. These vary very much. In early inquiries the amount of an antiseptic necessary to prevent putrefaction, *e.g.* in broth, urine, etc., was studied, but as bacteria vary in their powers of resistance, the method was unsatisfactory. It is now usual to estimate the effect of an antiseptic on pure cultures of pathogenic organisms, and in the case of sporing bacteria investigated. The

lyticus, *B. coli*, and the last being employed for testing the action on spores. A good method is to wash off the growth from a twenty-four hours' agar-slope culture, and suspend it in sterile distilled water, remove coarse particles by brief centrifuging and, rejecting the sediment, add a measured quantity of the supernatant suspension to a given quantity of varying dilutions of the antiseptic dissolved in distilled water. Then after the lapse of the period of observation, *e.g.* fifteen or thirty minutes, one or two loopfuls of the mixture are removed and placed in a great excess of culture medium; here it is preferable to use melted agar, which is then plated and incubated, since the number of colonies developing in comparison with the control, the *bactericidal* action even when medium is very comm

chemical agents it is necessary to be sure that the culture fluid is in great excess, so that the small amount of the antiseptic which is transferred with the bacteria may be diluted far beyond the strength at which it may be capable of inhibiting growth of the organisms. Sometimes it is possible at the end of the period of action to change the antiseptic into inert bodies by the addition of some other substance, but there is an objection to this procedure if a precipitate results, since the bacteria may be carried down with the precipitate and may escape the culture test. To test the effects of antiseptics on spores, Koch soaked silk threads in a suspension of anthrax spores and dried them. These were then subjected to the action of the antiseptic, well

washed in water, and laid on the surface of agar. In using this method to test the efficiency of mercuric chloride it was found necessary to treat the organisms with ammonium sulphide, otherwise the antiseptic effect of traces of the mercuric chloride fixed by the spores went on after they were removed to the culture medium. As a rule, the method described, in which the small amount of antiseptic adhering to the bacteria is diluted with an excess of culture fluid, can safely be followed, especially when a series of antiseptics is being compared. Krong and Paul introduced what is known as the 'garnet method' for testing antiseptics. In this, small glass beads of equal size are carefully cleaned, dipped in a suspension of spores of the anthrax bacillus, and allowed to dry. They are then placed in the antiseptic solution, and from time to time some are removed, washed, and well shaken in a measured quantity of water, this is plated, and the number of colonies developing is counted.

In order to test a slowly acting antiseptic, varying concentrations of the latter are added to a constant volume of fluid medium (peptone water, or serum sterilised by previous heating at 56°C), in a series of plugged sterile tubes, then the test amount of bacteria, e.g. a diluted young broth culture, is added to each; the tubes are incubated for twenty-four or forty-eight hours at 37°C and the presence or absence of growth noted by the appearance of turbidity and by subculturing. Another method consists in adding varying amounts of the antiseptic to a series of tubes, each containing a fixed amount of melted agar medium. The mixtures are then poured into Petri plates. When the medium is set and its surface thoroughly dry, a stroke inoculation is made with a dilute suspension of a young culture of the organism to be tested. The dilution aimed at is such that on the control plate, without antiseptic, the stroke will yield a line of semi-confluent colonies. In this way a number of different organisms can be tested on the same plate. The results are read after forty-eight hours' incubation at 37°C .

The standardisation of antiseptics. A watery solution of carbolic acid is commonly taken as the standard with which other antiseptics are compared. The procedure in *Rideal and Walker's method* is to inoculate with a fixed amount of bacterial suspension or of a twenty-four hours' broth culture, a fixed volume of a series of dilutions of the antiseptic to be tested, and at the same time a similar volume of a standard solution of chemically pure carbolic acid, e.g. 1 : 100, at short intervals (two and a half, five, seven and a half, and ten minutes) subcultures from all the tubes are made by inoculating tubes of broth with a loopful of each of the mixtures, and these cultures are kept at 37°C for forty-eight hours. The tubes containing the mixtures of antiseptic and bacteria are kept at a constant temperature (18° or 20°C) in a water-bath during the period of the test. The results show (1) the time required to produce sterility with the concentration of carbolic acid used (by means of a preliminary test, the concentration of carbolic acid is determined which kills the test organisms in seven and a half minutes, but fails to kill them in five minutes, this is the concentration to be used, and (2) the highest dilution of the other antiseptic which also produces sterility in the same time, say 1 : 3,000. The latter substance is then stated to have a 'carbolic acid coefficient' of 30, since under the conditions of the test the same bactericidal effect was produced by 1 : 100 carbolic acid. With a view to obtaining similar results in tests carried out on different occasions, it is usual to prepare the media under standard conditions as regards ingredients and pH, also, always to use the same strains of organisms (*B. typhosus* or *B. coli*), and to subculture them at fixed intervals, e.g. to maintain the stock culture by subculturing once a month, and for four or five days before the test to subculture

growth When water containing little or no organic matter, apart from bacteria, is placed in a vessel of silver, copper, etc., the organisms are killed, apparently by the traces of metallic compounds which pass into solution, the so-called *oligodynamic effect*

Among mercury compounds the *perchloride* is a strong antiseptic, a 1 : 1,000 solution being rapidly fatal to all non-spored organisms; and of all the salts of the heavy metals it has been most used. It is, however, a powerful poison for higher forms of life. Organic compounds of mercury have been introduced, e.g. *mercurochrome* (sodium salt of dibromoxy-mercury-fluorescein) and *merthiolate* (sodium ethyl mercurithio-salicylate), which, although powerfully antiseptic, are relatively non-toxic for mammalian tissues, it is probable that in these the activity resides in a complex ion

believed to act, in part at least, in virtue of reducing properties. *Formaldehyde*, used as formalin, a 40 per cent solution in water, is a powerful antiseptic and general poison. It is employed for treating infected articles, and, on account of its being volatile, is used for room disinfection, e.g. by spraying with 2 per cent. formalin. It tends to polymerise into inactive compounds

Phenol (*carbolic acid*), since its introduction for surgical purposes by Lister, has been much less powerful antiseptic than mercuric chloride, higher than phenol. It acts fairly well in vegetable solutions in vegetable solutions. A mixture of these with soaps is of great advantage of detergent as well as for treating infected articles. A solution of these in water is more effective than when dissolved

Krumwiede and Banzhaf recommend for the preservation of antiserum the addition of a mixture of equal parts of ether and cresol, so as to give a final concentration of 0.4 to 0.5 per cent of cresol, with immediate shaking to cause a uniform mixture. *Hexyl-resorcinol* belongs to this group. *Dettol* is a mixture containing halogen derivatives of xyleneols.

Basic organic dye-stuffs Certain of these are very powerful bacteriostatic agents, and the extremely low concentrations which at first inhibit the activity of the bacteria finally cause their death, e.g. crystal violet, brilliant green, the amino-acridine group (proflavine, acriflavine, 5-amino-acridine which is practically colourless), etc. The activity is enhanced in action by serum. They act best in mammalian tissues the 0.000 solution, as sur-violet (0.5 per cent

are important in leading to the mechanical removal of some bacteria from materials. Sodium and potassium salts of the saturated higher fatty acids, palmitic and stearic, have considerable bactericidal action on many organisms, including streptococci,

practically free the skin surface from the above-mentioned organisms. Water soap, chiefly composed of saturated acids, is the most active against typhoid bacilli, but acts powerfully on pneumococci and typhoid bacilli at ordinary concentrations. 0.000 solutions at a pH of 7.0 are effective for inactivation of Staphylococci

are usually less effective. A striking property of sodium lauryl sulphate is that when incorporated in agar they prevent the spreading of *B. proteus* without affecting growth at the site of inoculation, e.g. sodium lauryl sulphate, 1 : 10,000 (Lominski and Lendrum)

Alcohols Antiseptic action increases with the molecular weight from methyl to primary amyl alcohol. The presence of water is necessary for lethal action; and with ethyl alcohol the optimum addition is 30 to 50 per cent. While dilute ethyl alcohol kills spores at boiling point, they resist temperatures above 100° C. in the absence of water. Eighty per

uids such as serum. The antiseptic is finally removed by evaporation when a thin layer of the treated liquid is exposed to the air.

Aerosols and gases By aerosol is meant a mist of fine droplets, which can be produced by a suitable atomiser. Certain substances in this form, e.g. hypochlorite solution or a mixture of hexyl resorcinol and propylene glycol are highly effective in destroying bacteria in the air of rooms and at the same time can be breathed safely. Propylene glycol also, as vapour in the proportion of 1 gm. of the substance to from 2 to 4 million cubic centimetres of air, sterilises practically immediately the following organisms introduced into the atmosphere as suspensions in the form of fine spray—pneumococci, streptococci, staphylococci, Pfeiffer's bacillus, etc., and influenza virus (Robertson *et al.*)

Antibiotic agents—*Gramicidin, Penicillin, etc.* The term antibiotic is applied to an antiseptic substance which is a product of the growth of organisms. It has long been known that certain bacteria inhibit the growth of others *in vitro*. In the case of a solid medium this is demonstrated when a mixture of the two is used to inoculate the surface, no colonies of the susceptible bacteria develop close to one of the inhibitory kind, while at a greater distance there is growth of the former. Thus it has been found that *B. pyocyaneus* suppresses various organisms and a similar effect is produced by a sterile filtrate of a culture, 'pyocyanin', also, staphylococci inhibit the diphtheria bacillus and the typhoid bacillus is inhibited by certain strains of coliform bacilli. In some cases the explanation of the inhibition is simple, for instance, failure of the cholera vibrio to grow in the neighbourhood of *B. coli* on a medium containing a sugar fermented by the latter, is due to the resulting acidity, but often conditions are more complex. Further progress in this line was due to Dubos, who prepared from cultures of *B. brevis*, an aerobic sporing organism, an inhibitory product, *tyrothricin*; from this two alcohol-soluble polypeptides, gramicidin and tyrocidine were separated. Gramicidin in dilutions of upwards of one in a million *in vitro*, inhibits Gram-positive cocci, but does not lyse any organisms. Tyrocidine, which is somewhat less potent, acts on Gram-positive and, to a less degree, Gram-negative bacteria, provided proteins or peptones are absent. It also causes bacteriolysis of organisms which tend to autolyse readily. The action resembles that of bactericidal cationic surface-active agents. A number of other bacteria form antibacterial products, which have various chemical constitutions, and they are also produced by actinomycetes and fungi, e.g. *penicillin*, which will be treated under Chemotherapy.

From the examples which have been given it will be recognised that the choice of an antiseptic and the precise manner in which it is to be employed depend on the conditions under which the bacteria are to be killed. In practice it is essential to use an antiseptic in sufficient concentration, to let it act for sufficient time, and to ensure that it penetrates the material to be disinfected so as to come into contact with the bacteria. In illustration of the last point it may be mentioned that mercuric chloride solution will fail to disinfect sputum, because it coagulates the surface of the mass and so does not reach the interior.

Factors Modifying the Action of Antiseptics. The action of antiseptics depends upon various factors. Thus the medium in which the bacteria are suspended is important, most of the powerful antiseptics are greatly diminished in their action by the presence in the fluid of proteins or minute particles of organic matter which, according to Chick and Martin, act as adsorbing agents, and this is the reason why the action of such antiseptics on bacteria in faeces or sputum is limited in degree. The presence of water in the medium also plays an important part; thus phenol or mercuric chloride when dissolved in alcohol is a much less powerful antiseptic than when in watery solution and the same is true of phenol in a vegetable oil; and 50 to 70 per cent. alcohol kills organisms more rapidly than absolute alcohol. Again,

the *temperature* at which the mixture of antiseptic and organisms is kept has a great influence; e.g. with *B. paratyphosus* at temperatures from 0° C. to 35° C. a rise of 10° C. increases the rate of killing by HgCl₂ three- to four-fold and by phenol seven- to fifteen-fold. Other factors, such as the hydrogen-ion concentration, the degree of ionisation, and the dispersion of the antiseptic, may have a marked effect upon the lethal action of a given substance on bacteria. The effect of *hydrogen-ion concentration* is well illustrated by the basic amino-acridine compound, acriflavine, which has a much more powerful antibacterial action at a high pH (9) than at a low (5). The presence of NaCl, which reduces the *ionisation* of mercuric chloride, diminishes the antiseptic power. On the other hand, phenol, the antiseptic effect of which is due to the molecule as a whole, is increased in its action by the presence of salt. As regards the influence of *dispersion*, Chick and Martin found that certain antiseptics of the phenol group acted more powerfully when in the form of a fine emulsion than when in true solution, the former condition apparently favouring adsorption of the antiseptic by the bacteria.

The killing of bacteria or spores by an antiseptic appears as a rule to proceed like a chemical reaction 'of the first order', such as the inversion of sugar by acid; thus, when the antiseptic is in excess, the number of organisms killed in a given time bears a fixed proportion to the number present at the beginning of the period of observation—the logarithmic law. Variation in concentration of the antiseptic affects the rate of killing to a degree which depends on the particular substance. Thus Chick found that a 1·2 per cent solution of phenol killed a suspension of paratyphoid bacilli in 3·5 minutes, but with 0·6 per cent phenol 240 minutes were required, i.e. when the concentration was halved the killing time was increased seventy times. On the other hand 1:1,000 HgCl₂ killed the organisms in 1·5 minutes and 1:2,000 HgCl₂ took 7 minutes, i.e. halving the concentration increased the killing time only five times.

Antiseptic properties, of course, depend essentially on chemical constitution. The chemical characters which determine the antiseptic properties of substances are, however, complex and still to a great extent obscure. Some antiseptics, such as phenol or mercuric chloride, act rapidly, and concentrations which fail to kill the bacteria within a few hours may have little effect in inhibiting their multiplication subsequently. On the other hand, some basic organic dyes are only slowly lethal, at first they merely prevent proliferation of the organisms (bacteriostatic action), and twenty-four or forty-eight hours are required for the development of their maximum lethal effect. Another property of certain antiseptics is their *selective action*, for instance, with crystal violet Gram-positive organisms are in general more susceptible than Gram-negative, the concentration required to kill *B. coli* is five hundred times greater than that which kills staphylococcus. Both of these organisms, however, are practically equal in their susceptibility to mercuric chloride. In comparing different antiseptics it is important to express their activity in terms of the molecular concentrations of the solutions used.

Mode of Action of Antiseptics. The bactericidal agencies originally

a general property of protoplasm poisons. Later it appeared that many antiseptics do not coagulate proteins and also that in some cases the lethal effect can be counteracted by treatment with suitable antidotes for a considerable time after the antiseptic has been fixed by the organisms.

Accordingly an antiseptic may combine with some particular constituent of the bacteria, an essential metabolite or some enzyme, and thereby bring to an end processes necessary for multiplication of the cells, as is postulated in the case of mercury acting on the SH groups. Further, selective action is frequently a marked feature, one species of organism tolerating even many hundred times the concentration which kills another. In this respect there is often parallelism between the behaviour to antiseptics and to Gram's stain, Gram-positive organisms being highly susceptible to certain antiseptics, such as hexamethyl violet and cationic surface-active substances, which have little action on the Gram-negative bacilli of the coli-typhoid group. Among members of the same group of organisms too, marked differences may exist, such as the relative resistance to brilliant green shown by typhoid and paratyphoid bacilli as compared with the common intestinal types of coliform bacilli. When the lethal action occurs gradually, so as to enable its progress to be followed numerically, it is usually found that throughout the greater part of the course the number of organisms or spores killed in a unit period of time is a constant proportion of those alive at the beginning of the period. This is analogous to the chemical 'law of mass action', where one reactant (here the bactericidal agent) is present in great excess. The explanation advanced is that the resistance of the bacteria fluctuates rhythmically, so that only a proportion are susceptible at any given instant (Chick). Finally, antiseptic properties are exhibited by substances so diverse chemically that it appears highly unlikely that they can have a uniform mode of action, illustrative examples have already been given. As regards the mechanism of the killing action, fixation of the antiseptic by the organisms is the first stage; this may be accompanied by concentration of the substance in or on the organisms in virtue of adsorption or surface action or owing to the affinity of particular chemical constituents of the organisms for the active ions of the substance. This last mechanism is well illustrated by antiseptics of the amino-acridine group, acriflavine etc. Here it is the cation which produces the bactericidal effect since this is intensified when the pH is increased (Browning *et al.*). Presumably the cation combines with nucleic acid components of the organisms, as nucleic acid or nucleotides reverse the antiseptic action (McIlwain). It has been found also in this series that increase in basicity runs parallel up to a certain point with increase in antiseptic potency, as well as hydrophilic property (Rubbo *et al.*). At present, however, leaving out of consideration those agents which denature proteins, it is seldom possible to identify the mechanism of lethal action as consisting in interference with any particular anabolic or katabolic process of the organisms (*vide* chapter on Chemotherapy).

THE CLASSIFICATION OF BACTERIA

What is said under this heading will be chiefly confined to the characters of the pathogenic bacteria. In the past there have been numerous schemes set forth for the broad classification of bacteria, all based in general on the same fundamental principles. There has been little agreement, however, among systematists as to the characters on which more detailed classification should be based, and even yet our knowledge of the essential morphology and relations of certain bacteria is too limited for an exact classification on a strictly biological basis. Identification of species is dependent, not only on the morphology of individual organisms and that of growths on culture media, but also on physiological and biochemical characters, pathogenicity to animals under experimental conditions, and in some cases on delicate serum

reactions. In recent years an extensive scheme of classification has been put forward by American systematists, and this will be referred to below after a simple general account has been given.

The division into Lower and Higher Bacteria can be recognised, though transitional forms have to be accounted for. In subdividing the bacteria

chaëtes. Subsidiary, though important, points in the further subdivision are the planes in which fission takes place among the cocci, the presence or absence of spores, etc. The recognition of actual species is often a matter of great difficulty. The points to be observed in this will be discussed in connection with the individual organisms

I. THE LOWER BACTERIA. These, as we have seen, are minute unicellular masses of protoplasm surrounded by an envelope, the total vital capacities of a species being represented in every cell. They present the four distinct forms mentioned above. Endogenous sporulation may occur, they may also be motile.

1. *Cocci* (or *Micrococci*). In this group the cells range in different species from $0.5\ \mu$ to $2\ \mu$ in diameter, but most measure about $1\ \mu$. Before division they may increase in size in all directions. The main groups are usually classified according to the method of division. If the cells divide only in one axis, and remain attached, then a chain of cocci will be formed. The group in which this occurs is known as a *Streptococcus*. If division takes place irregularly, the resultant mass may be compared to a bunch of grapes, and the group is called a *Staphylococcus*. Division may take place in two axes at right angles to one another, in which case cocci adherent to each other in plates of four (called tetrads) or multiples of four may be found, the former number being the more frequent. This form of coccus has sometimes been designated *Tetracoccus*. The individuals in a growth of micrococci often show a tendency to remain united in twos. This form is spoken of as a *Diplococcus*, but may not be distinctive, since every coccus as a result of division becomes a diplococcus, though in some species the tendency to remain in pairs is well marked. In some species capsules are a characteristic feature—often these have a well-marked outer limit, e.g. pneumococcus, sometimes they are of great extent, the diameter being many times that of the coccus (e.g. *Streptococcus mesenteroides*). In none of the cocci have endo-

cubes of eight with the sides which are in contact slightly flattened. Large numbers of such cubes may be lying together. The sarcinae are, as a rule, rather larger than the other cocci. Most of the cocci are non-motile, but a few motile species possessing flagella have been described, possibly, however, these are more of the nature of short bacilli—cocco-bacilli.

2. *Bacilli*. These consist of long or short cylindrical cells, with rounded or sharply rectangular ends, usually not more than $1\ \mu$ broad, but varying very greatly in length. They may be motile or non-motile. Where flagella occur, these may appear to be distributed all round the organism—*peritrichous*, or only at one or both of the poles—*terminal*. Several species are provided with sharply marked capsules (e.g. pneumobacillus). In many species endogenous sporulation occurs. The spores may be central, terminal, or subterminal, round, oval, or spindle-shaped. There is no doubt that among the bacilli in certain cases, e.g. *B. tuberculosis*, the phenomenon of

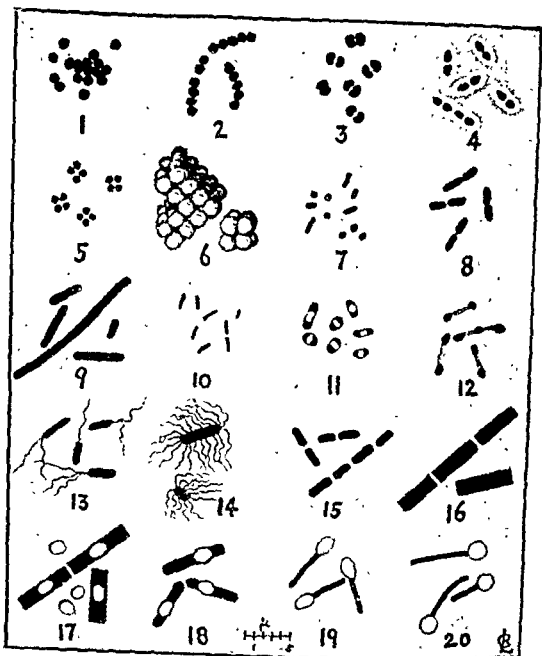


FIG. 3 1-6. Different types of cocci. 7-20. Different types of bacilli
17-20. Spore-bearing types of bacilli All $\times 2,000$

1. Spherical cocci, single, diplococcal and clustered forms (Cocci)

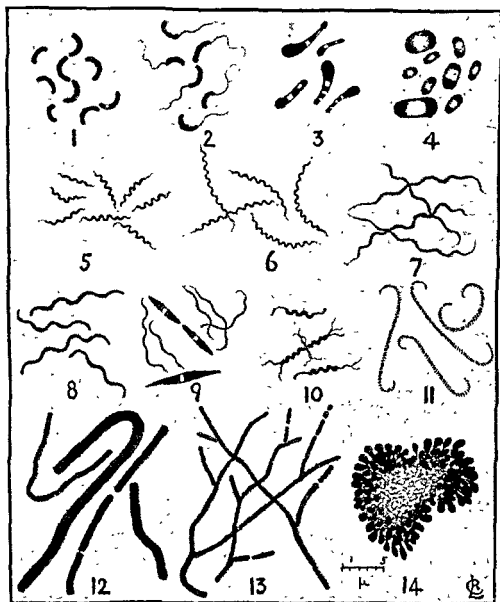


FIG 4

true branching may occur. Such instances form a connecting link between the bacilli and the higher bacteria, *e.g.* streptothrices.

3. *Vibrios* and *Spirilla*. These consist of curved rods or cylindrical non-flexuous cells more or less spiral or wavy. The unit is usually a short curved rod or vibrio (often referred to as a 'comma' form). When two or more of the latter occur, as they often do, end to end with their curves alternating, then a wavy or spiral thread results. An example of this is the cholera organism. The motile species possess terminal flagella. Of the latter there may be one or two, or a bunch containing as many as twenty, at one or both poles. Division by transverse fission takes place as among the bacilli.

4. *Spirochaetes*. These are elongated flexuous cells which show undulations or true spirality. As a rule they are slender in proportion to their length, and some of the smaller forms are of extreme tenuity. Motility has generally been regarded as due to the contractility of the protoplasm and may be of a lashing, wave-like, oscillatory, or corkscrew-like character. Recently, by special methods of staining and also by means of the electron microscope, definite flagellar structures have been demonstrated in certain spirochaetes. The larger forms reach a considerable size and a relatively great length, and some of them have a spirally-twisted crest running along the whole length of the cell. They multiply by transverse fission, but longitudinal division has been described in some of them, although this is doubtful. The pathogenic species belong to different genera—*Treponema*, *e.g.* organisms of syphilis and yaws, etc., *Borrelia*, *e.g.* organisms of relapsing fever, and *Leptospira*, *e.g.* organism of infective jaundice. The morphological characteristics of these are described in later chapters.

The above forms are illustrated in Figs. 3 and 4.

II THE HIGHER BACTERIA. These consist generally of filaments, branched or unbranched. In most cases the filaments at more or less regular intervals are cut by septa into short rod-shaped or curved elements. Such elements are more or less interdependent, and special staining methods are often necessary to demonstrate the septa which demarcate the individuals of a filament. There is often a definite membrane or sheath common to all the elements in a filament. Not only, however, is there this close organic relationship between the elements of the higher bacteria, but there is also interdependence of function, for example, one end of a filament is frequently concerned merely in attaching the organism to some other object. The greatest advance on the lower bacteria, however, consists in the setting apart among some of the higher bacteria of the free terminations of the filaments for the formation of reproductive *conidia*; groups of such conidia may be formed by division in three different planes.

The conidia have a free existence for a certain time before becoming attached, and in this stage are sometimes motile. They are usually oval or rod-like, sometimes pyriform. They do not possess any special powers of resistance.

The following may be given as a brief summary of the main groups: (a) The *Chlamydobacteria* or *Trichobacteria* are filamentous forms, occurring mainly in water, and are usually sheathed; the sheath is often impregnated with iron oxide. They are often sessile, and the attached ends thinner than the distal. Conidium formation is often met with in the free ends of the filaments, the conidia may be motile or non-motile. True branching does not occur, but false branching is sometimes met with, due to a terminal cell becoming laterally displaced, *e.g.* *Cladothrix*. (b) A further development occurs in the *Streptothrices*, to which the *Actinomyces* and other pathogenic

species belong (Fig. 4, 13). Here the organism consists of a felted mass of non-septate filaments in which true dichotomous branching occurs. Under certain circumstances threads grow out and produce chains of conidia, from which new individuals can be reproduced. These conidia are sometimes spoken of as spores, but they have not the same staining reactions nor resisting powers of so high a degree as the endospores of bacteria. Sometimes, too, the protoplasm of the filaments breaks up into bacillus-like elements which may also have the capacity of originating new individuals. In the *Actinomyces* a club-shaped formation at the end of the filament may occur, the nature of this structure will be discussed in a later chapter (Fig. 4, 14). The *Streptothrix* group may be regarded as a link between the bacteria on the one hand and the lower fungi on the other. Like the latter, it shows the felted mass of branching filaments which is usually called a mycelium, whilst the breaking up of the protoplasm into coccus- and bacillus-like forms links it to the true bacteria. (In medical bacteriology the name *Leptothrix* has generally been applied to elongated filamentous organisms with morphological similarities to the *Streptothrices* but differing from these in being unbranched—Fig. 4, 12). (c) The *Thiobacteria* or sulphur bacteria are characterised by the presence of sulphur granules in their protoplasm. bacterio-purpurin also may be present. Some of them are sheathed and attached, and form conidia, e.g. *Thiothrix*, others again are elongated, sheathless, and show an undulatory motion due to the contraction of the protoplasm, e.g. *Beggiatoa*.

Classification and Nomenclature of American Systematists. Owing to the unsatisfactory state of bacterial classification and nomenclature, an endeavour has been made by American systematists to develop a system of classification in accordance with that adopted in other branches of biological study. The classification advocated is sufficiently elastic to provide for modification as knowledge of the relationships of the bacteria is added to. This system has also introduced a new nomenclature and eliminated the trinomial and clumsy designations that had crept into bacteriology. However, such terms as gonococcus, meningococcus, *Bacillus typhosus* or typhoid bacillus, etc., are likely to maintain their traditional use in preference even to designations that clearly indicate the approved biological genus and species of the organisms. The new classification, nevertheless, serves a most useful purpose in its orderly grouping together of biologically allied species.

The bacteria are collectively designated as the class of *Schizomycetes*

spherical, cylindrical, comma-shaped, spiral, filamentous, or united into filamentous, flat, or cubical aggregates, filamentous aggregates often surrounded by a common sheath, filaments may show true or false branching, reproducing by simple fission, in some species resting bodies in the form of endospores, reproductive conidia produced by some filamentous forms, cells in certain species motile and possessing flagella.

The class of SCHIZOMYCETES is divided into the following orders

1 EUBACTERIALES (true bacteria). Undifferentiated simple forms, spherical or rod-shaped, straight or curved, or spiral filaments, no true branching, some motile due to flagella, non flexuous, multiplying by transverse fission, some produce endospores, no conidia, some species pigmented, some store volutin, glycogen, or fat, include many disease-producing species.

2 ACTINOMYCETALES. Rod-shaped or filamentous 'mould-like' organisms with a tendency to branch and produce mycelium (e.g. *Actinomyces*); often showing 'club'

formations; without spores, but producing conidia in some species; mostly Gram-positive; non-motile, some are parasites of animals and plants; aerobic, but some anaerobic or micro-aerophilic; growth slow.

3 CHILAMYDOBACTERIALES. Filamentous aquatic forms which may show false branching; include the 'iron bacteria', which are 'alga-like' sheathed organisms, the sheath containing iron oxide

4 MYXOBACTERIALES Include the myxobacteria, organisms exhibiting a pseudoplasmodial and a resistant cyst-forming phase; 'slime-mould' like in nature

5 SPIROCHÆTALES 'Protozoon-like' in certain characters; usually relatively slender, flexuous, spiral filaments, multiplying apparently in some species by longitudinal fission, though transverse fission is the characteristic mode of division as in other bacteria

It may be noted that in this scheme the first order constitutes most of the *lower bacteria* as above described, whilst orders 2, 3, and 4 would represent the *higher bacteria*. The organisms pathogenic to the human subject belong to the orders 1, 2, and 5.

The genera of importance in medical bacteriology are briefly described in the following summary; type-species are also given; for reference to these see the general text of the book. For full details of this system of classification Bergey's *Manual of Determinative Bacteriology*, 6th edition, should be consulted

Additional orders recently suggested are. **RICKETTSIALES** (the organisms named *Rickettsia* and *Bartonella*, and described in Chap XXVI; and **VIRALES** (the filterable viruses)

ORDER EUBACTERIALES

Families

NITROBACTERIACEÆ Motile or non-motile, non-sporing and usually Gram-negative bacillary forms, characterised by their capacity to utilise simple inorganic compounds. The nitrifying bacteria of soil belong to this family. None of these organisms is pathogenic

RHIZOBIACEÆ Rod-shaped cells with single polar or lateral flagellum or 2 to 4 peritrichous flagella, or non-motile, usually Gram-negative, utilising glucose and sometimes other sugars but without apparent acid production, include the nitrogen-fixing organisms which occur in the root nodules of the *Leguminosæ*

PSEUDOMONADACEÆ Straight or spirally curved rod-shaped or filamentous organisms, usually motile with terminal flagella, non-flexuous, Gram-negative

ACHROMOBACTERIACEÆ Gram-negative rod-shaped organisms of uniform shape, motile with peritrichous flagella or non-motile, non-chromogenic, or pigmented (yellow, brown, or orange), non-saccharolytic or feebly saccharolytic, water or soil saprophytes, plant pathogens, or animal parasites

AZOTOBACTERIACEÆ Large rod-shaped or oval cells, utilise free nitrogen, the free-living nitrogen-fixing bacteria belong to this family

MICROCOCCACEÆ Non-sporing and for the most part non-motile spherical or globose forms, usually Gram-positive

NEISSERIACEÆ Gram-negative aerobic or anaerobic cocci, generally obligate parasites

STREPTOBACTERIACEÆ (or **LACTOBACTERIACEÆ**) Gram-positive spheroidal or rod-shaped cells tending to occur in chains, ferment sugars and other carbohydrates with abundant production of organic acids

PARVOBACTERIACEÆ Small Gram-negative rod-shaped forms; aerobic or anaerobic; usually obligate parasites; not actively saccharolytic.

ENTEROBACTERIACEÆ Gram-negative rod-shaped forms, frequently motile with peritrichous flagella, active fermenters of various carbohydrates with acid or acid and gas production.

CORYNEBACTERIACEÆ Mostly non-motile Gram-positive rod shaped organisms; showing barred or beaded staining and often metachromatic granules, aerobic or micro-aerophilic, animal or plant parasites, also occur in soil, water, and dairy products

BACTERIACEÆ Miscellaneous non-sporing rod-shaped organisms not included in other families

BACILLACEÆ Rod-shaped cells characterised by the formation of endospores, generally Gram-positive, aerobic or anaerobic

FAMILY PSEUDOMONADACEÆ

Genera of medical importance :

- Pseudomonas*. Principally water and soil bacteria producing a water-soluble diffusible pigment (green, blue, or yellowish-green) ; motile or non-motile bacillary forms , Gram-negative, e.g. *B. pyocyaneus*
Vibrio Rigid curved rod-shaped forms, occurring singly or united into spirals , motile with usually a single terminal flagellum usually Gram-negative, e.g. *V. cholera*
Spirillum Rigid spiral organisms, which are usually motile and possess multiple terminal flagella , mostly water organisms

FAMILY ACHROMOBACTERIACEÆ

Genus of medical importance

- Alcaligenes* Non-chromogenic , Gram-negative bacillary forms occurring as commensals in intestine of man and animals , do not ferment carbohydrates and do not liquefy gelatin , e.g. *B. faecalis alcaligenes*

FAMILY MICROCOCCACEÆ

Genera

- Micrococcus* Saprophytic or facultatively parasitic cocci, which occur in plates or irregular masses , generally Gram-positive , some species produce yellow, orange, or red pigment , the type species is *M. luteus* (This genus also includes the staphylococci)
Gaffkya Gram-positive cocci which tend to be arranged in tetrads, e.g. *Micrococcus tetragenus*
Sarcina Cocci arranged in regular bundles of eight or multiples of eight

FAMILY NEISSERIACEÆ

Genera

- Neisseria* Gram-negative cocci in pairs , aerobes , e.g. meningococcus
Veillonella Gram-negative cocci occurring in masses, rarely in pairs , anaerobes , e.g. *Veillonella parvula*

FAMILY STREPTOBACTERIACEÆ (OR LACTOBACTERIACEÆ)

Genera of medical importance

- Diplococcus* Gram-positive cocci uniformly arranged in pairs , capsules well-developed , bile-soluble , e.g. pneumococcus
Streptococcus Gram-positive cocci arranged in chains but sometimes in pairs , capsules may be present , bile-insoluble
Lactobacillus Gram-positive, non-motile, rod shaped forms, which are frequently long and slender , produce abundant acid from sugars , some are thermophilic tend to be micro-aerophilic , occur in milk , e.g. *B. acidophilus*

FAMILY PARVOBACTERIACEÆ

Genera

- Pasteurella* Parasitic Gram-negative bacillary forms tending to exhibit bipolar staining, usually non-motile , aerobes , associated with hæmorrhagic septicæmia in animals, and plague in man , e.g. *B. pestis*
Brucella Gram-negative, non-motile bacillary or cocco-bacillary forms which are pathogenic to man and animals , some species require for primary growth in media an increased carbon dioxide concentration , do not ferment carbohydrates , gelatin is not liquefied , e.g. *B. abortus*
Malleomyces Rod-shaped forms, sometimes forming threads and showing tendency to branching , motile or non-motile , Gram-negative , gelatin may be liquefied , parasitic in animals and man , e.g. *B. mallei*
Hamophilus Parasitic, non-motile, Gram-negative pleomorphic bacillary organisms, which tend to be relatively minute , grow aerobically and best (or only) in the presence of hæmoglobin, and in general requiring blood serum, ascitic fluid, or certain growth factors , e.g. *B. influenza*

Noguchia Minute, slender, Gram-negative, rod-shaped forms present in the con-
motile,
perature,

Dialister Minute, non-motile, Gram-negative parasitic bacillary organisms which grow only under anaerobic conditions and in media containing fresh tissue or serum; occur in respiratory tract; e.g. *Bact. pneumosintes*.

Actinobacillus Aerobic, Gram-negative bacillary forms which show pleomorphism; pathogenic to animals, in tissues form aggregates resembling colonies of *Actinomyces* with characteristic clubs (qv); grow best under increased tension of carbon dioxide, carbohydrates fermented with acid but no gas production, e.g. *Actinobacillus*

Bacteroides Motile or non-motile, non-sporing, anaerobic Gram-negative bacilli occur-
fragilis
acteristic tapering

Fusci

Morax Gram-negative; aerobic, growing best in presence of blood or serum; liquefying coagulated serum, parasitic or pathogenic in conjunctiva; e.g. *Diplobacillus* of Morax.

FAMILY: ENTEROBACTERIACEÆ

Genera of medical importance

Escherichia Gram-negative, aerobic bacilli, commonly present in intestinal tract of mammalian animals, some are motile and possess peritrichous flagella; fermentation of carbohydrates, particularly glucose and lactose, with acid or acid and gas formation is a characteristic feature, but gelatin is not generally liquefied; e.g. *B. coli*

Aerobacter This genus has been differentiated from *Escherichia*, to which it is closely related, by its negative methyl red reaction and its production of acetyl-methyl-carbinol from glucose as indicated by the occurrence of the Voges-Proskauer reaction. Such organisms may also occur in the intestinal tract. The genus includes *B. aerogenes*. These organisms are non-motile, tend to produce an abundant viscid growth, and can also be differentiated from the typical *B. coli* by their fermentation of inositol and their capacity to utilise salts of organic acids (e.g. sodium citrate). It is doubtful if this supposed genus can be separated biologically from that designated *Klebsiella*, and its separation from *Escherichia* is also somewhat artificial.

Klebsiella Short, Gram-negative, encapsulated bacilli, aerobic; ferment various carbohydrates, occur as parasites in the upper respiratory passages and may be associated with inflammatory lesions in this region; e.g. *pneumobacillus*

Proteus Saprophytic and parasitic, Gram-negative, pleomorphic bacilli which are motile and possess peritrichous flagella; grow aerobically and tend to produce a 'spreading' growth on culture medium; ferment glucose but not lactose; usually liquefy gelatin, e.g. *B. proteus*

Salmonella Parasitic, Gram-negative, aerobic, usually motile bacilli associated with inflammatory conditions of the intestine of man and animals; ferment various carbohydrates but not lactose or saccharose; do not usually liquefy gelatin. This genus is exemplified by the typhoid and paratyphoid bacilli, and *B. enteritidis* of Gaertner

Shigella Similar to *Salmonella* but invariably non-motile, and usually non-gas-producing in the fermentation of carbohydrates, e.g. the dysentery bacilli

Serratia Aerobic bacillary organisms usually producing a red pigment on culture media, e.g. *B. prodigiosus* (or *Serratia marcescens*)

FAMILY CORYNEBACTERIACEÆ

Corynebacterium Parasitic, Gram-positive, non-motile, rod-shaped organisms, with a tendency to club-shaped forms and may show branching in old cultures; stain unevenly, non-acid-fast, aerobic, e.g. *diphtheria bacillus*

Listeria Motile, Gram-positive, rod-shaped forms with single terminal flagellum; aerobic or micro-aerophilic, pathogenic to man and animals; infection characterised by mononucleosis, e.g. *Bact. monocytogenes*

Erysipelothrix Gram-positive rod-shaped forms with tendency to develop filaments which may branch, aerobic or micro-aerophilic; usually parasitic; e.g. *Bacillus* of swine erysipelas

FAMILY *BACILLACEÆ*

Genera :

- Bacillus*. Gram-positive, sporing, bacillary forms which tend to occur in chains and form rhizoid colonies; mostly saprophytes, generally liquefy gelatin; e.g. *B. subtilis*. The anthrax bacillus belongs to this genus.
- Clostridium*. Like *Bacillus* but anaerobic or micro-aerophilic; many species are parasitic and pathogenic; e.g. *B. tetani*.

ORDER *ACTINOMYCETALES*

Families of medical importance :

ACTINOMYCETACEÆ Characterised by their filamentous elements, which are often branched and interlaced, forming a mycelium, conidia are sometimes produced, some species are parasitic.

MYCOBACTERIACEÆ. Rod-shaped forms which are only occasionally branched or filamentous, conidia are not produced; the protoplasm often stains unevenly.

FAMILY : *ACTINOMYCETACEÆ*

Genera :

- Actinomyces* Typical filamentous forms which branch and form mycelium, but may segment into shorter bacillary or coccid elements, sometimes parasitic and pathogenic, when growing in tissues the colonies may show radial, club-shaped structures at their periphery, some species are anaerobic or micro-aerophilic; e.g. *Actinomyces*.
- Nocardia* Slightly branched, sometimes more or less branched and forming a mycelium, but later assuming rods and club-shaped structures, some species are pathogenic, Gram-positive, may be fastidious, may occur by budding as well as fission, many species pigmented, e.g. *Actinomyces farcinicus* (*Nocardia farcinica*).

FAMILY . *MYCOBACTERIACEÆ*

Genus :

- Mycobacterium* Non-motile, rod-shaped forms which stain with difficulty but when stained are acid-fast, club-shaped forms are sometimes observed and occasionally also elongated and branched elements, aerobic, growth on culture medium is often slow, several species are pathogenic; e.g. tubercle bacillus.

ORDER *SPIROCHÆTALES*

Genera of medical importance

- Borrelia* These are parasitic spirochætes with coils of relatively long wave-length and usually three to five in number. They are fairly refractile under the microscope and are readily stained with the usual aniline dye solutions. The relapsing fever spirochætes are examples of this genus.
- Treponema* Parasitic forms with undulating or rigid spirilliform body, the coils being of relatively short wave-length, and often showing great regularity. They are feebly refractile and not readily stained by the usual dye solutions. The spirochæte of syphilis is classified in this genus.
- (It should be noted that the spirochætes comprising these two genera, *Borrelia* and *Treponema* are sometimes classified together and designated by the generic name *Treponema*.)
- Leptospira* Parasitic and saprophytic forms consisting of sharply twisted filaments and with one or both ends 'hooked' or recurved; feebly refractile and difficult to stain by the usual dye solutions, the coils are fine and numerous (seen by dark-ground illumination); differ from the other groups in their ability to survive in water; e.g. *Leptospira icterohæmorrhagiae* of infectious jaundice.

CHAPTER II

RELATIONS OF BACTERIA TO DISEASE—INFECTION— PRODUCTION OF TOXINS BY BACTERIA

IN most cases, before micro-organisms produce disease they must obtain a foothold in the tissues or blood, multiply and carry on their vital processes there. These acts of invasion and multiplication are called 'infection'. The disturbances in structure and function in the host which result in consequence of infection by a parasite constitute the infective disease. An essential feature of such diseases and one which accounts for their very manifold nature is that the etiological factor is living. Accordingly in the interaction between the host and the parasite two living agents are concerned each of which is capable of considerable adaptation. The factors on the part of the host which tend to antagonise the parasite are summed up in the term 'resistance'—the converse being 'susceptibility'. The capacity of the organism to produce disease is described as 'virulence'. It has already been stated that a strict division of micro-organisms into true *parasites* and *saprophytes* cannot be made. No doubt there are organisms, such as the tubercle bacillus, gonococcus, etc., which are in natural conditions always parasites associated with disease. But these can lead a saprophytic existence in specially prepared conditions, e.g. artificial culture media, and there are many of the disease-producing organisms, such as those of enteric fever and cholera, which can survive in nature under favourable conditions. A similar statement applies to the terms *pathogenic* and *non-pathogenic*. By the term pathogenic is meant the power which an organism has of producing morbid effects in the animal body, either under natural conditions or in artificial conditions experimentally arranged. We know of no organism which will in all circumstances produce disease in all animal species, and, on the other hand, many bacteria described as harmless saprophytes can produce pathological changes if introduced in sufficient quantity. The term pathogenic, therefore, is merely a relative one, and indicates that in certain circumstances the organism will produce disease, though it is often used in Medicine as implying that the organism produces disease in man under *natural* conditions. The term '*commensal*' is applied to organisms which flourish normally on the skin or mucous surfaces of the body. Some commensals, e.g. staphylococci and streptococci, have the capacity under certain conditions of invading the tissues and producing pathogenic effects. This is a matter of importance in connection with the origin of natural infections.

It will be shown more fully below that many of the pathogenic bacteria, as regards their morphological and cultural characters, are very similar to organisms normally present in the sites at which the former invade the body. Thus the typhoid-paratyphoid and dysentery groups are related to *B. coli* (the common commensal of the intestines), and the meningococcus to various Gram-negative diplococci which abound in the naso-pharynx. This suggests strongly that in their evolutionary history the specific pathogens have been derived from commensals, but the circumstances under which such transformation has taken place are unknown, and it has not been effected under experimental conditions. A few organisms are known which are capable of invading the body and even multiplying in the blood, but which appear to

be practically devoid of pathogenic action, e.g. *Spirillum minus* in the mouse and *Trypanosoma lewisi* in the rat. A similar behaviour is shown also in infection of the rat with the leptospira of infectious jaundice. In the mouse, inoculation with *Tr. pallidum* produces no lesions or other manifestations of disease, but the spirochaetes persist for long periods in the body and they invade the central nervous system.

Koch's Postulates. In regard to the proof of a micro-organism being specifically pathogenic and causal of a particular disease, certain requirements, generally described as Koch's postulates, may be mentioned here. These are (1) that the organism should be found constantly present in cases of the disease, (2) that it should be isolated from the disease in pure culture, (3) that artificial cultures so obtained should be able to reproduce the disease in experimental animals, and (4) that the organism should again be isolated from the experimentally infected animals. Some writers add to these that specific antibodies should be demonstrable in the blood of the diseased person or animal. In Koch's historical studies of the tubercle bacillus the four postulates mentioned were fulfilled, thus providing irrefutable proof of the causal rôle of the organism. The same requirements can be satisfied for the anthrax bacillus and certain other pathogenic organisms. But in many diseases it has not been possible to establish complete proof of causal relationship according to these strict rules. Thus in the case of typhoid fever, a man with cultures do not develop in an subject, and yet all other being the causal agent of typhoid fever.

Infection. As explained above, infection implies the successful invasion of the tissues or blood by a micro-organism which establishes itself there, multiplies actively and as a rule produces morbid effects. It must be recognised that bacterial invasion is not in all cases successful, in that the organism may be unable to establish itself and bring about an obvious pathological condition. Thus it is probable that certain commensal bacteria from mucous membranes may frequently invade the lymphatics and even the blood, e.g. *B. coli*, staphylococci and streptococci, but under normal conditions are destroyed by the natural defences, while specific pathogenic organisms may produce some degree of infection without apparent clinical or pathological effects. It has been customary to refer to this state of infection without disease as *subinfection* or *subclinical infection*.

Infection is sometimes classified into (1) *endogenous* and (2) *exogenous*. In the former case the source of the infection is in the body itself, the causal organism having previously existed as a commensal. Thus, infection of a wound by staphylococci from the skin would be described as endogenous.

from another person or animal harbouring it, such person or animal being either the subject of the particular infective disease or an apparently healthy 'carrier' (*vide infra*). It must be noted specially that in many instances such transmission of pathogenic organisms is quite indirect, thus, spores of the anthrax bacillus derived from cases of the animal disease may be deposited on soil, persist there for a time, and ultimately infect other animals, or may be transmitted to man by wool, hides, etc. Again, the spores of the tetanus bacillus introduced into a wound may appear to be derived from soil, but in reality originate in the intestines of animals.

Sources and Modes of Infection. The sources and modes of exogenous infection are exceedingly varied and a proper understanding of the propagation of an infective disease requires a full knowledge of (1) the way in which the specific organism is discharged from the body of an infected person or animal, (2) the viability of the organism apart from its human or animal host, and (3) the avenue or avenues by which it invades the body of a new host. The bearing of such knowledge on our understanding of infective diseases will be fully illustrated in later chapters. It will suffice here to exemplify the varied sources and modes of infection which have to be dealt with in such diseases.

(1) *Routes of discharge of organisms.* Certain organisms are discharged from the human body in the secretions of the respiratory tract, *e.g.* the tubercle bacillus, pneumococcus, *Streptococcus pyogenes*; others in the excreta, *e.g.* the typhoid-paratyphoid bacilli in the faeces and urine, and the dysentery bacilli in the faeces. The mode of spread of these two categories of pathogenic bacteria respectively is, therefore, profoundly different. Bacteria from the respiratory passages may be discharged not only in actual sputum, but also in the droplets of secretion sprayed into the air by the acts of sneezing, coughing, or even speaking. The larger of such droplets rapidly settle by gravity and the bacteria in them may contaminate the dust of premises; the smaller tend to remain suspended in the air and by evaporation form 'droplet-nuclei', so that air-borne infection is rendered possible. This subject will be discussed further in Chapter XXXVII. With regard to infections transmitted from animals, the mode of discharge of the organism from the animal body is also obviously of importance: thus, tubercle bacilli from infected dairy cows are discharged, often in large numbers in the milk as a result of udder disease; the virus of rabies is present in the saliva of an infected dog, and is thus transmitted by the bite of the animal.

(2) *Viability outside the host* Some micro-organisms are strictly parasitic and cannot survive long outside the body of their host, *e.g.* the gonococcus, and when this is the case transmission is usually by direct contact. Others can retain their viability for some time apart from their host, and may be transmitted by 'vehicles' of infection, *e.g.* by water in the case of the typhoid bacillus and the cholera vibrio. But the conditions under which such organisms are placed often determine their viability. Although the cholera vibrio may remain alive for a time in water, it dies rapidly if subjected to drying. Similarly the meningococcus cannot withstand the drying influence of the atmosphere, while other organisms survive for varying times under such conditions and can be transmitted readily by air and dust, *e.g.* the tubercle bacillus and *Streptococcus pyogenes*.

Particular species of microbes are biologically adapted to a habitat in blood-sucking insects and other arthropods, and thereby are transmitted from their human or animal hosts by these 'vectors'. Thus, the plague bacillus flourishes in the stomach of certain fleas, the *Rickettsia* of typhus fever grows in the lining cells of the gut of lice. Some micro-organisms may actually go through a phase of their life cycle in insects, *e.g.* the malaria *Plasmodium* in mosquitoes. In other cases insects may act as vectors in a more mechanical way; thus, the house-fly may convey mechanically on its legs and body the organisms of enteric fever, dysentery, and cholera from faeces to food materials; but it has been shown also that when this insect has fed on excreta containing typhoid or paratyphoid bacilli, these organisms may persist in its alimentary tract for a few days.

(3) *Avenues of infection.* The avenue of infection is through a body surface, the skin, or a mucous membrane. The skin with its dense epidermis presents normally a barrier to infection, though some organisms are able to

invade even the intact skin, e.g. certain pathogenic fungi and *Leptospira icterohæmorrhagiae*; and it is possible that hair follicles may provide a means of access for certain species, e.g. staphylococci, the anthrax bacillus, etc. On the other hand, a wound obviously offers an easy avenue for bacterial invasion.

In considering the way in which bacteria enter the body by dermal invasion through the skin, it must be remembered that while some bacteria can flourish on this surface of the body, others seem to die when placed on the skin. This suggests that the dermal secretions have bacteriostatic or bactericidal properties, but so far these have not been defined. It may be, however, that the low pH, viz. 5.2 to 5.8, is a factor in this respect (Arnold *et al.*)

Mucous membranes with their thinner epithelium and numerous ducts and follicles provide easier entrance to micro-organisms, but it has to be recognised that these membranes possess a certain degree of protection against bacterial invasion through the mechanical action of the secretions flowing from the ducts and washing the surface. The action of the ciliated epithelium of the respiratory mucosa also exerts some mechanical protection. In addition certain secretions contain chemical or biological factors which are antagonistic to various bacteria, e.g. the hydrochloric acid of the gastric juice, and bactericidal constituents which are possibly analogous to those contained in normal serum and which will be referred to later in the chapter on Immunity. A substance of this nature called 'lysozyme' has been demonstrated in certain mucous secretions, particularly tears, but is active mainly against saprophytic cocci. In some cases the respiratory tract constitutes the chief route of infection, e.g. in tuberculosis and pneumococcal infection, and various organisms may enter through the tonsils, nasal, pharyngeal, or naso-pharyngeal mucosa, e.g. streptococci, meningococcus. In other diseases the digestive tract is the avenue, as exemplified in enteric fever and dysentery. Certain organisms enter the body by the urogenital system, e.g. gonococcus.

From a general study of infective diseases it is clear that frequently successful invasion is restricted to a particular avenue, depending on the specialised parasitic properties of the specific organism. Thus the cholera vibrio is able to establish itself by invasion of the alimentary tract after being swallowed, but cannot do so if introduced into the subcutaneous tissues. Virulent streptococci capable of producing an acute infection *via* a wound may be harmless if introduced into the stomach and intestine. In this connection also various bacterial species and viruses show a special 'tropism' or affinity for particular tissues once they have invaded the body. This will be referred to later.

Carriers. As has been stated, many of the organisms which produce inflammatory and suppurative affections are normally present on the skin or mucous membranes, but it has also been established that several of the causal agents of acute infectious diseases, such as enteric fever, epidemic meningitis, diphtheria, etc., may flourish in the bodies of individuals in apparent health. Such individuals are known as 'carriers', and they play a highly important part in the spread of these infections. One group of carriers is constituted by those who have suffered from the disease, and in whom the organism persists after recovery, and these are usually designated 'convalescent' carriers, and 'temporary' or 'chronic' according to the duration of the condition. The proportion of those who become carriers, and the period of carriage, vary in different diseases. In cholera, for example, the period is usually comparatively short, whereas in enteric fever the organisms not infrequently persist for an indefinite period of time. Also, especially

in a number of protozoal infections, *e.g.* piroplasmosis, after recovery from the acute attack the organisms persist permanently in the blood; this state has been termed non-sterilising immunity or 'premunition' (Sergent and Parrot). In the human subject, malaria and certain virus infections may present similar features. The other group of carriers, sometimes designated 'paradoxical', comprises apparently healthy individuals who harbour the organism but are not known to have suffered from the disease. Some of these may really have had a mild attack, but in others there is no evidence of this. A few subsequently develop the disease—'precocious' carriers, although the carrier state may occur temporarily without a clinical attack of the disease developing. In many instances previous contact with a case of the disease can be traced—'contact' carriers. On the other hand, no connection of this kind may be discoverable; for instance, the meningococcus is often found, especially during epidemics, in 'non-contacts', the organism apparently spreading widely from individual to individual in the community. This illustrates a very important principle regarding the spread of infective diseases. The causal organism may be transmitted through several insusceptible individuals in whom it persists or even multiplies for a time without causing recognisable illness, but such persons are capable of infecting a susceptible individual and setting up a typical attack. This explains the failure to establish continuity between successive cases which is often a feature of epidemics. Frequently chronic carriers exhibit some local pathological condition, *e.g.* chronic cholecystitis with gall stones in the biliary carrier of typhoid or paratyphoid bacilli. Animal carriers may also be sources of human infection, *e.g.* dairy cows with *B. abortus* in their udders and milk, rats carrying *Leptospira icterohæmorrhagiae* in their kidneys and discharging this organism in their urine. Further facts will be given in connection with particular diseases.

Modifying Conditions. In studying the pathogenic effects in any instance, the animal affected as well as the micro-organisms must be considered, and not only the species of each, but also its exact condition at the time of infection. In other words, the resulting disease is the product of certain characters of the subject of infection, on the one hand, and of the infecting agent, on the other. We may, therefore, state some of the chief circumstances which modify each of these two factors involved, and, consequently, the condition of disease produced.

1. The Subject of Infection. Here we shall consider only individuals who have not previously been infected with the micro-organism in question, since such earlier contact with the organism or its products may bring about specific changes in reactivity, which are dealt with later in the chapter on Immunity. Among healthy individuals susceptibility to a particular organism may vary according to (a) species, (b) race and individual peculiarities, (c) age. Different species of the lower animals show the widest variation in this respect, some being extremely susceptible, others highly resistant, *e.g.* the susceptibility of equines to the glanders bacillus and the resistance of cattle. There are parasites, such as that of syphilis, which under natural conditions are peculiar to the human subject and produce disease experimentally in only a few of the lower animals. There are others, such as the gonococcus with which the typical lesions can be produced in animals, or appear only imperfectly, whose pathogenic effects may follow injection of cultures.

Resistance to experimental infections has been investigated by Webster, Schütze and others, who have shown that different lines of mice may vary markedly in their susceptibility to intraperitoneal inoculation with organisms

of the *Salmonella* group, while manifesting no differences to similar infection with other organisms, *e.g.* *Pasteurella* group, or the pneumococcus, etc. In the human subject, differences in susceptibility to a certain disease are found amongst different races, and also amongst individuals of the same race, as is well seen in the case of tuberculosis. Age plays an important part, young subjects being more liable to certain diseases, *e.g.* fungus infections of the hair (ringworm). Where the susceptibility to an infection decreases with age, however, as in the case of diphtheria, there is the possibility that a part may be played by the specific factor of exposure to the infective agent and the development of immunity through 'subclinical' infection. On the other hand, at very early ages there may be a relative insusceptibility. For instance, Burky found that young rabbits withstood a larger dose of staphylococcus toxin than mature animals, *e.g.* the mothers. Children under two months old show a similar insensitiveness of their skin to injections of killed staphylococci (Kobak and Pilot). Further, at different periods of life certain parts of the body are more susceptible, for example, in early life the bones and joints to tuberculous and acute suppurative infections. In some cases, however, resistance to an infective disease in the early months of life may be due to passive immunity derived from the mother. This is exemplified by

followed in the train of famine. Experimental proof has also been obtained that exposure to abnormal temperatures, physical fatigue, and malnutrition may lower resistance to bacterial infection. As already explained, the intact skin and mucous membranes act as an efficient protection against the entrance into the deeper tissues of many organisms, in fact, the latter seem often to be actively destroyed on the surface. But a local susceptibility may be produced by injury or diminished vitality. As examples of the effect of mechanical injury, if, previous to an intravenous injection of staphylococci, the aortic cusps of a rabbit be injured, the organisms may settle there and set up an ulcerative endocarditis, if a bone be injured, they may produce suppuration at the part, whereas in ordinary circumstances these lesions would not take place. The work of Findlay has suggested that on injury to the tissues there is liberation locally of histamine-like substances which lead to dilatation of the capillaries along with increased permeability, so that organisms in the circulation will pass out and localise in the area. The local effect of certain tissue extracts, *e.g.* testicular extract (Duran-Reynals), in increasing the permeability of the tissues to bacteria and toxins may be a similar phenomenon. Severe trauma of the tissues is an important factor in predisposing to wound infections, particularly tetanus and gas gangrene. In this case necrotic tissue and blood-clots in the wound provide a favourable nidus for the germination of spores and multiplication of the organisms, as will be explained in Chapter XX. It is of special interest that in certain circumstances mucin has a marked influence in exalting the infective properties of various bacteria, thus, a dose of meningococcus culture which by itself would be practically non-pathogenic when inoculated into the peritoneum of a mouse, may lead to a rapidly fatal septicæmia if injected along with pig's gastric mucin. The underlying biological factors involved and the significance of the phenomenon have not yet been clearly defined.

It has been recognised clinically that alcoholic intoxication may predispose to certain infections, *e.g.* pneumonia, and the observations of Pickrell

afford experimental evidence in support of this view ; thus intoxication of animals with alcohol destroys their resistance to the pneumococcus, even

anæsthesia.) The part played by these local defences will be referred to later.

In addition to such conditions as have been mentioned, there are also some special factors which predispose to infection. In the case of silicosis of the lungs, a marked liability to infection with the tubercle bacillus has long been recognised, and it has been shown by Kettle that silica has a special effect in making the tissues susceptible to invasion by this organism. Another striking example is the remarkable effect which ionisable calcium salts have in leading to infection with the *B. welchii* and other anaerobes, as was first observed by Bullock and Cramer. Thus, when *B. welchii*, free of its toxin, is injected into a mouse or guinea-pig it rapidly becomes destroyed by lysis or by phagocytosis, whereas if a solution of calcium chloride is injected along with the organism or its spores, a spreading gas gangrene results. A similar effect may follow, though not so readily, when the calcium salt is injected at some part of the body other than the site of injection of the bacilli. In contrast to this, salts of magnesium, sodium, potassium, etc., are devoid of such favouring effects. Calcium salts accordingly seem to have a special action in breaking down the defences of the tissues or leading to a state of 'kataphylaxis', as these workers have called it. These results suggest that other specific factors as yet unknown may be concerned in the origin of infections occurring naturally.

Apart from the effect of the state of general nutrition on susceptibility to infections, a special position is occupied by vitamin A. When animals are fed for a considerable period on food which lacks this accessory factor, they tend to develop spontaneous pyogenic infections such as xerophthalmia, abscesses in the mouth, nasal sinuses and middle ear, broncho-pneumonia, enteritis, and infections of the urinary tract : that is, the surface commensals are apparently enabled to invade the tissues. A diet deficient in this vitamin causes alterations in the epithelium lining ducts and acini of glands, which becomes keratinised ; and it is suggested that the consequent obstruction of ducts by masses of these desquamated cells offers a favourable nidus for bacterial growth and so is responsible for the infection. Lassen has shown, however, that there is also a general diminution in resistance, since rats which have developed signs of vitamin A deficiency are more susceptible to subcutaneous or intravenous inoculations of a *Salmonella* group organism than are normal or under-nourished animals. The change is attributed to functional impairment of the reticulo-endothelial system. According to Green and Mellanby, the addition of carotene to diets lacking vitamin A restores the anti-infective property. Vitamin C deficiency seems to predispose to infection of mucous membranes, as is illustrated in scorbutic conditions ; and the observations of Glazebrook and Thomson on the incidence of pneumonia and acute rheumatism among boys in a training establishment in which there was a vitamin C dietary deficiency, indicate the influence of this vitamin in resistance to certain infections. Harde and others have found that ascorbic acid protects guinea-pigs against an otherwise fatal dose of diphtheria toxin. There is also some evidence that deficiency in the vitamin B complex may lower resistance. No loss of resistance can definitely be attributed to deficiency in vitamin D. It seems likely from all the data available that deficiency of various dietary constituents may adversely

influence resistance to infection, but the experimental evidence on this subject is quite incomplete. Apart from organic substances, certain minerals and salts may be important factors, and it is of interest that inorganic elements such as iron and copper may have a dietary rôle in maintaining natural resistance (Perla).

A notable example of diminished resistance to infection as a result of a metabolic disorder is seen in diabetes; tuberculosis and infection by pyogenic bacteria are prone to occur in this condition and are apt to be of a severe character. It has also been shown that rats, which are naturally immune to glanders, can be rendered susceptible by being fed with phloridzin which produces a form of diabetes. The possibility of other endocrine disorders being responsible for increased susceptibility to infection must be borne in mind though at present definite evidence on this question is lacking.

Such facts, established by experiment (and many others might be given), illustrate the important part which local or general conditions of diminished vitality or disordered metabolism may play in the production of disease in the human subject. In normal conditions the blood and tissues of the body, with the exception of the skin and certain of the mucous surfaces, are bacterium-free, and if a few organisms gain entrance, they are destroyed. But if the vitality becomes lowered, their entrance becomes easier and the possibility of their multiplying and producing disease greatly increased. In this way the favouring part played by fatigue, cold, etc., in the production of bacterial diseases can be understood. It is important to keep in view in this connection that many of the pyogenic organisms are normally present on the skin and various mucous surfaces. It is not uncommon to find in the bodies of those who have died from chronic wasting disease, collections of micrococci or bacilli in the capillaries of various organs, which have entered in the later hours of life—that is to say, due to invasion from body surfaces, the bacterium-free condition of the blood has been lost in the period of prostration preceding death. It should also be remembered that the action of one species of organism may devitalise the tissues to such an extent as to prepare the way for the entrance of other bacteria; we may mention the liability to the occurrence of pneumonia, erysipelas, and various suppurative conditions, in the course of, or following, the specific infectious diseases. In some cases the primary organism or virus may produce lesions through which other organisms gain entrance, e.g. in enteric fever, diphtheria, influenza, etc.

In the course of infections there is a tendency for the body to alter in its behaviour towards the micro-organisms and their products. Thus altered reactivity or *allergy* manifests itself in various ways and frequently takes the form of supersensitiveness, *i.e.* intensification of the phenomena of damage or reaction. The view has been advanced that in those infective diseases characterised by a long incubation period, part of this time is occupied by

the former being relatively mild, the latter severe as regards destructive effects :

Again, in
ages has

and it is impossible to say at present how far the usual manifestations of

a later occasion react more rapidly and more intensely to contact with the same micro-organism (or its products) and that this may result in preventing the latter from again obtaining a foothold.

The methods by which the natural resistance may be specifically increased belong to the subject of *immunity*, and are described in the chapter on that subject.

2. The Infecting Agent. In the case of a particular species of bacterium, its effect will depend chiefly upon (a) its virulence, and (b) the number introduced into the body. To these may be added (c) the path of infection.

The *virulence*, i.e. the power of invading the tissues or blood, multiplying and producing disease, varies greatly in different conditions, and the methods by which it can be diminished or increased will be described later (*vide* Chapter III). Natural barriers which are effective against organisms of low virulence, e.g. the skin and mucous membranes, may be penetrated, with the setting up of infection, by the same species when highly virulent. One important point is that when a bacterium has been enabled to invade and multiply in the tissues of an animal, its virulence for that species is often increased. The virulence sometimes may be still more increased by inoculating from one animal to another in series—the method of *passage*. Widely different effects are, of course, produced on the virulence being altered. For example, a streptococcus which produces merely a local inflammation or suppuration, may produce a rapidly fatal septicæmia when its virulence is raised. Virulence also has a relation to the animal employed, as occasionally on being increased for one species of animal it is diminished for another. For example, streptococci, on being inoculated in series through a number of mice, acquire increased virulence for these animals, but become less virulent for rabbits. Certain facts suggest that there may be a periodicity in virulence, i.e. that an organism may for a time produce a relatively mild type of disease and then develop into a more potent strain capable of overcoming the resistance of a greater number of individuals, this would account for the fact that in the case of some diseases widespread epidemics occur at intervals of years. It may be said that under natural conditions there are two important properties of an organism upon which its virulence depends, (1) *invasiveness* or *aggressiveness*, i.e. capacity to invade the tissues and flourish in the body of the host, and (2) *toxigenicity*, or power to produce toxins or poisons which cause damage to the tissues. These two properties are not necessarily correlated and may be independent of one another; thus the tetanus bacillus has only slight invasive action, but its virulence is high in virtue of its great toxigenic power. The anthrax bacillus on the other hand is extremely invasive but its toxic properties are not relatively great. The aggressiveness of an organism as a factor in its virulence is clearly correlated with its power of overcoming the local and general defences of the body, and can best be studied in relation to immunity mechanisms and the individual characters of the various pathogenic organisms. It may, however, be mentioned here that in some species, e.g. the pneumococcus and the anthrax bacillus, capsule formation is an important factor in virulence and that loss of the property of capsulation deprives the organism of its virulence. The chemical substance of which the capsule is composed is thus protective against certain mechanisms of defence. Biological changes due to variation, e.g. in some cases the 'smooth' to 'rough' transformation of colonies already described in Chapter I, may be associated with loss of virulence, such variation being also associated with alterations in the antigenic constitution of the organisms, i.e. the loss of chemical constituents present in the original pathogenic strain. In certain species, e.g. the typhoid bacillus, a particular constituent (Vi antigen) has been

defined as a virulence factor. These subjects are more fully discussed in later chapters, and also the problem of degree of toxigenicity as a factor in virulence.

The *number* of the organisms introduced, *i.e.* the dose of the infecting agent, is another factor of prime importance. The healthy tissues can usually resist a certain number of pathogenic organisms of moderate virulence. It is only in a few instances that one or even several organisms introduced will produce a fatal disease, *e.g.* the case of anthrax in white mice. The healthy peritoneum of a rabbit can resist and destroy a considerable number of pyogenic cocci, but if a larger dose be introduced, a fatal peritonitis may follow. There is, therefore, for a particular animal, a minimum lethal dose which can be determined by experiment only—a dose, moreover, which is modified by various circumstances difficult to control.

The *path of infection* may determine the result. Staphylococci injected subcutaneously in a rabbit may produce only a local abscess, while on intravenous injection multiple abscesses in certain organs may result and death may follow. Again, with a particular species of host, *e.g.* the mouse, one strain of streptococcus may be highly virulent when introduced into the nasal cavity, but not when injected into the subcutaneous tissue, whereas with most virulent strains the opposite is the case (Loewenthal). Local inflammatory reaction with subsequent destruction of the organisms may take place entirely at the site of infection or may occur also in the related lymphatic glands. The latter therefore act as a second barrier of defence, or as a filtering mechanism which aids in protecting against blood infection. This is well illustrated in the case of septic infections of wounds. In some other conditions, however, the organisms are very rapidly destroyed in the blood stream, thus Klemperer found that in the dog subcutaneous injection of the pneumococcus produces death more readily than intravenous injection. As has already been shown, the route by which bacteria enter the body under natural conditions varies according to the particular organism. Certain organisms are also highly selective in their affinities for particular tissues or organs. Thus the site of primary infection by the gonococcus is restricted to the genito-urinary tract or the conjunctiva, while the pneumococcus most commonly infects the respiratory tract. On the other hand, many organisms such as the tubercle bacillus and the pyogenic cocci may attack various parts. It is these affinities which determine the infectivity of secretions and excretions of affected individuals. Of course, we are ignorant as to the exact factors which are responsible for an organism flourishing, say, in one mucous membrane and not in another. Invasion of the tissues may occur rapidly, as is seen in the case of *Tr. pallidum*, which, after being applied to scratches on the scrotal skin of the guinea-pig, has been found in the popliteal lymph glands five minutes later.

Mixed infection with several species of organisms is sometimes of importance in determining the final results of a microbic disease. One of the most striking examples of this is swine influenza, which is due to infection with a bacillus similar to Pfeiffer's influenza bacillus together with a filterable virus. Neither of these agents by itself is highly pathogenic, whereas the two together produce a severe condition. More commonly a second organism becomes superadded to a pre-existing infection, especially when a free surface is involved, and so aggravates the primary disease, *e.g.* infection with pyogenic organisms additional to tuberculosis in the lungs where there is cavity formation. In mixed infections, the biological phenomena of symbiosis and antibiosis probably play some part. In the condition of Vincent's angina, two apparently different species are regularly associated in symbiotic relationship, a spirochæte and fusiform bacillus. The growth of the influenza

bacillus is assisted by the presence of other organisms, *e.g.* staphylococci, and such symbiosis may be operative in mixed infections of the respiratory passages by these organisms. Antibiotic effects may also come into play in mixed infections, *e.g.* in wounds.

Modes of Bacterial Action. In the production of disease, organisms there are two main modes of action, *(a)* the production of the living organism, and *(b)* the production by them of poisons or toxins which may act both upon the tissues locally and upon the body generally. One of these processes may be the more prominent feature, but both are always more or less concerned.

1 *Multiplication and spread of bacteria in the body.* After pathogenic bacteria have invaded the tissues, their further behaviour varies greatly in different cases. Either the organisms remain local, in which case they may produce little tissue change, as in tetanus, or a well-marked lesion, as in diphtheria, etc. Even though the organisms remain localised, effects on remote parts of the body or generalised effects may be produced by the circulation of their toxins in the blood (*toxæmia*). On the other hand, the bacteria themselves may pass by the lymph or blood stream to other parts or organs in which they settle, multiply, and produce lesions, as in tuberculosis. In certain cases they may multiply in the blood stream, producing a septicæmia. In the lower animals this multiplication of the organisms in the blood throughout the body may be very extensive, *e.g.* the septicæmia produced by the pneumococcus in rabbits. But in septicæmia in man it is in a less degree, the organisms seldom enter the blood, and their detection and even methods of culture are negative results unless a considerable amount of blood is used. But in such cases the organisms may be found *post mortem* lying in large numbers within the capillaries of various organs, *e.g.* in cases of septicæmia produced by streptococci. However, in infections by certain spirochætes (relapsing fever) and protozoa (malaria, etc.) the causal organisms may occur in large numbers in the peripheral blood. It is important to draw a distinction between the mere presence of bacteria in the blood—*bacteriæmia*, and their active multiplication in it—*septicæmia*. The former condition, which is frequent in many infections, represents merely an overflow of the organisms from the lesions, as is well exemplified in the early stages of typhoid fever. It is also a common occurrence in subacute infective endocarditis where organisms may be cultivated from the blood over a considerable period. But there is no progressive multiplication in such cases, and we may say that in bacteriæmia the organisms would soon disappear if the source of supply were removed. Where a chronic focus of infection exists, however, the continued dissemination of organisms and their products may cause serious effects either upon the body generally or on particular tissues.

2 *Production of poisons or toxins.* The growth of the organisms is accompanied by the formation of chemical products which act locally or generally in varying degree as toxic substances. The toxic substances become diffused throughout the system, and their effects may be manifested by symptoms such as fever, disturbances of the circulatory, respiratory, and nervous systems, etc. In some cases associated changes in the tissues are found, *e.g.* those in the nervous system in diphtheria, to be afterwards described. The general toxic effects may be so slight as to be of little importance, as in the case of a local suppuration; or they may be very intense, as in septicæmia and pyæmia, or, again, less severe but producing cachexia by their long continuance, as in tuberculosis.

The occurrence of local tissue changes or lesions, as already mentioned, is

one of the most striking results of bacterial action. These also are due, directly or indirectly, to substances formed by the bacteria. But in this case it is more difficult to demonstrate the mode of action, for in the tissues chemical products are formed by the bacteria slowly, continuously, and in a certain degree of concentration, and these conditions cannot be exactly reproduced by experiment with products obtained in artificial cultures. Further, it is very doubtful whether all the chemical substances formed by a certain bacterium growing in the tissues are also formed by it in cultures outside the body. The separated toxin of the diphtheria bacillus, like various vegetable and animal toxins, produces a local toxic action of very intense character, manifested often by extensive necrotic change. It has also to be noted that more than one toxin may be produced by a given organism, e.g. *Staphylococcus aureus*, *Streptococcus pyogenes*, or the tetanus bacillus.

The injection of large quantities of many different pathogenic organisms in the *dead* condition results in the production of a local inflammatory change which may be followed by suppuration, this effect being possibly brought about by certain substances in the bacterial protoplasm common to various species. When dead tubercle bacilli, however, are introduced into the blood stream, nodules result in certain parts, which have a resemblance to ordinary tubercles. In this case the bodies of the bacilli evidently contain a highly resistant and slowly acting substance which gradually diffuses and produces its effects (*vide* Tuberculosis).

To sum up, we may say that the action of bacteria as disease-producers depends upon the chemical products formed directly or indirectly by them. This action is shown by *tissue changes* produced in the vicinity of the bacteria or throughout the system, and by *toxic symptoms* of great variety of degree and character. The degree and nature of these changes and symptoms may

afterwards the nature of the chemical products.

EFFECTS OF BACTERIAL ACTION

These may be for convenience arranged in a tabular form as follows

A Tissue Changes

- (1) Local lesions, i.e. changes produced in the neighbourhood of the bacteria

Position (a) At primary focus

(b) At secondary foci

Character : (a) Degeneration or necrosis } Acute or chronic
(b) Tissue reactions

- (2) General lesions, i.e. changes produced at a distance from the bacteria, directly or indirectly, by the absorption of toxins

(a) In special tissues—

(i) degeneration or necrosis, e.g. in secreting cells, vessel walls, nerve cells and fibres

(ii) changes of a reactive nature in the blood-forming tissues and organs

(b) General pathological changes, the effects of malnutrition or of increased waste.

B Disturbances of Metabolism

The occurrence of fever, of errors of assimilation and excretion, etc

C Symptoms

A. Tissue Changes. The effects of bacterial action are so various as to include almost all known pathological changes. However varied in character, they may be classified under two main headings: (a) those of a degenerative or necrotic nature, the direct result of damage; and (b) those of reactive nature, defensive or reparative. The former are the expression of the essential vulnerability of the tissues, the latter of protective powers evolved for the benefit of the host. In the means of defence both leucocytes and certain cells of the tissues are concerned. Both show phagocytic properties, *i.e.* have the power of taking up bacteria into their protoplasm. The cells are attracted to the focus of infection by chemotaxis, and thus we find that different bacteria may attract different cells. The most rapid and abundant supply of phagocytes is seen in the case of suppurative conditions where the neutrophile polymorphonuclear leucocytes of the blood are chiefly concerned. When the local lesion is of some extent there is usually an increase of these cells in the blood—a neutrophile leucocytosis. Moreover, observation has shown that associated with this there is in the bone marrow an increased number of the precursors of these leucocytes—the neutrophile myelocytes. The facts abundantly show that defence is not a mere local mechanism, and that increased proliferative activity in distant tissues is called into play.

In other cases the cells chiefly involved are the large mononuclear leucocytes, endothelial cells and histiocytes, these form together a group of non-granular cells to which Metchnikoff gave the name *macrophages*, and which more recently have been designated the *reticulo-endothelial system*. These cells are all characterised by their capacity to ingest minute particles, such as bacteria or finely divided suspensions of carbon, iron oxide, etc., and they also store in granular form certain colloids presented to them in solution, such as the 'vital' stains. The cells which possess this phagocytic property are especially the endothelia lining lymph gland sinusoids and the blood sinuses in the spleen, liver, and bone marrow, also the reticulum cells of lymphatic tissue, spleen, and bone marrow, the histiocytes of connective tissue in general, and a proportion of the monocytes in the circulating blood; less active in this respect are the endothelia of other blood and lymph vessels and the fibrocytes. When living bacteria such as pneumococci are injected into the blood stream, they are rapidly taken up by these phagocytes, the subsequent events depending on the virulence of the organisms. If they are weakly virulent, they may permanently disappear from the circulation, being phagocytosed and eventually destroyed, mainly in the endothelial cells of the spleen which, as it were, filters them out of the circulation, and also in the Kupffer cells of the liver, etc., but in the case of a virulent strain they reappear again in the blood and, in the severest form of infection, progressively increase till death occurs from septicæmia. It is clear that such phagocytes play an important part in the fixation and destruction of bacteria; also, on the analogy of their behaviour towards foreign colloids, it is thought that they may fix circulating toxins similarly. On the other hand, it has not been possible with certainty to produce marked increase in the susceptibility to organisms which cause general infections, by exclusion of a considerable part of the reticulo-endothelial system. Such attempts have taken the form either of splenectomy or of 'blockade' by repeated massive injections of substances which are actively stored by the phagocytic cells, or both procedures have been combined. Except in certain infections which are greatly increased in severity after splenectomy, such as that with the spirochæte of relapsing fever, these procedures have not led to definite lowering of resistance. One point which must be borne in mind here is that the proliferative capacity of

the cells of the reticulo-endothelial system is so great that the chances of effecting extensive blockade are remote. On the other hand, it has been demonstrated by Ledingham that a local increase of such cells may enhance resistance; thus an area of skin in which the phagocytic cells were present in excess owing to previous infiltration with India ink, failed to develop the usual lesions on the intracutaneous injection of the vaccinia virus at this site. Similarly, the resistance of serous sacs to infection can be intensified by procedures which lead to a local accumulation of macrophages. Reaction by such cells is well seen in typhoid fever, where the specific bacillus appears to have little or no effect on the neutrophile leucocytes. In other cases, again, the reaction may be mainly on the part of the connective tissue cells, although their proliferation is always associated with some variety of leucocytic infiltration and usually also with the formation of new blood vessels. The leucocytic infiltration may consist of large numbers of lymphocytes, but the function of these cells in reactive processes is still imperfectly understood. Such connective tissue reactions occur especially in chronic infections or in the later stages of an acute infection. The reactive tissue changes in the presence of bacterial invasion are naturally very varied—examples of this will be found in subsequent chapters—but they may be said to be manifestations of the two fundamental processes of (a) increased functional activity—exudation from blood vessels, phagocytosis, etc., and (b) increased formative activity—cell growth and division. The exudation has been variously interpreted, however. There is no doubt that the exudate may have bactericidal or opsonic properties and it also acts as a diluting agent, but it must still be held as uncertain whether the process of exudation ought to be regarded as primarily defensive or as the direct result of damage to the endothelium of the vessels. It may also be pointed out that none of the various changes referred to is peculiar to bacterial invasion; they are examples of the general laws of tissue change under abnormal conditions, and they can all be produced by chemical substances in solution or in a particulate state. What constitutes their special feature is their progressive or spreading nature, due to the bacterial multiplication.

(1) **LOCAL LESIONS** In some diseases the lesion has a special site—for example, the lesions of enteric fever in the lymphoid follicles of the small intestine. In other cases it depends entirely upon the point of entrance, e.g. malignant pustule of anthrax and wound infections due to pyogenic bacteria. In others again, there is a special tendency for certain parts to be affected, as the upper parts of the lungs in tuberculosis.

When organisms gain entrance to the blood from a primary lesion, the organs specially liable to be affected vary greatly in different diseases. The meningococcus shows a 'selective affinity' for the pia-arachnoid membrane. Pyogenic cocci have a special tendency to settle in the capillaries of the kidneys and produce miliary abscesses, whilst these lesions rarely occur in the spleen. On the other hand, the nodules in disseminated tuberculosis or glanders are much more numerous in the spleen than in the kidneys, which in the latter disease are usually free. Thus the distribution of lesions cannot be explained always on a mechanical basis. Organisms, either bacteria or protozoa, which have reached the circulation, tend to accumulate in the pulp of the spleen, causing a proliferative reaction of the cells, as well as necrosis of the Malpighian bodies sometimes. This, together with the removal of damaged cells from the circulation, leads to enlargement of the organ, which may be marked in some acute infections such as enteric fever. In an infection of old standing, such as chronic malaria, fibrosis occurs also, and the enlargement may be great. Similarly, the lymphatic glands may undergo some

general enlargement in both acute and chronic infections when the organisms are widely distributed in the body, even apart from their presence in considerable numbers in the gland tissue.

The question also arises as to whether particular strains of organisms may have a selective affinity for certain organs or tissues. According to Rosenow, for example, cultures of streptococci isolated from gall-bladder disease in man when injected intravenously into animals such as rabbits, tend to localise specially in the gall-bladder. General confirmation of this view is lacking, but it appears that sometimes such may be the case. Thus, among many cultures of *B. coli* Helmholz found several which when injected intravenously into rabbits produced chiefly renal lesions.

Acute local lesions. The local inflammatory reaction presents different characters in different conditions. It may be accompanied by abundant fibrinous exudation, by great catarrh (in the case of a mucous surface), by hæmorrhage, or by cedema; it may be localised or spreading in character; it may be followed by suppuration, and may lead to necrosis of the tissues, a good example of the latter event being a furuncle. Among the pyogenic organisms staphylococci and streptococci habitually show a different behaviour on inoculation into the same species of animal. Thus staphylococci tend to produce large localised suppurations, while the hæmolytic streptococci cause diffuse spreading inflammations. In order to elucidate this difference, Menkin injected a vital dye, trypan-blue, into infected areas of skin in rabbits; he found that with the staphylococcus there was blocking of the lymphatics of the part within an hour. After inoculation with the streptococcus, however, the lymphatics remained permeable for thirty hours. Such differences probably depend on the characters of the toxins and enzymes produced by these organisms. Other examples will be given in subsequent chapters, but it will be seen how complex may be the factors on which the production of a characteristic lesion depends. The necrotic or degenerative changes affecting especially the more highly developed elements of tissues are chiefly produced by the direct action of the bacterial poisons, though aided by the disturbances of nutrition involved in the vascular phenomena. It may here be pointed out that a well-marked inflammatory reaction is often found in animal species which occupy a medium position in the scale of susceptibility; and also that an organism which typically causes a general infection in a certain animal species, may produce only a local inflammation when its virulence is lessened. The lymphatic glands which drain the site of a local lesion tend to enlarge, there being a hyperplasia of the lymphoid cells, with which is often associated an infiltration of neutrophile leucocytes—lymphadenitis. These changes are partly due to organisms which reach the glands, but also to products formed at the site of the primary infection. Sometimes, as in bubonic plague, the lymphadenitis is not accompanied by any lesion at the site of infection.

The production of lesions in particular cells and tissues by viruses, as well as the special structures termed inclusion bodies, constitute problems which can be discussed more appropriately in a later chapter.

Chronic local lesions. In a considerable number of diseases produced by bacteria the local tissue reaction is a more chronic process than that described above; there is less vascular disturbance and a greater preponderance of the proliferative processes, leading to new formation of granulation tissue. This formation may occur in scattered foci, so that nodules result, or it may be more diffuse. Such changes especially occur in the diseases often known as the *infective granulomata*, of which tuberculosis, syphilis, leprosy, glanders, actinomycosis, etc., are examples.

Neoplastic changes. Such changes have not been found to be directly associated with bacterial infection. Rous showed, however, that a sarcoma in fowls is due to a filterable virus and since then a number of infective tumours in birds have been described. In mammals of several species papillomata are caused by filterable viruses and recently it has been found by Rous that an infective papilloma of cotton-tail rabbits, studied by Shope, when transferred to tame rabbits, causes lesions which ultimately become the seat of epithelioma. So far, however, it has not been demonstrated that a virus is essential to the continued growth of this malignant tumour.

(2) **GENERAL LESIONS PRODUCED BY TOXINS** In the various infective conditions produced by bacteria changes commonly occur in certain organs unassociated with the actual presence of the organisms, these are due to the action of bacterial products circulating in the blood. Many such lesions can be produced experimentally. The specialised cells of various organs, particularly the kidney and liver, are liable to changes of this kind. Cloudy swelling, which may be followed by fatty change or by actual necrosis with granular disintegration, is common. Hyaline change in the walls of arterioles may occur, and in certain chronic conditions amyloid degeneration is brought about in a similar manner. The latter has been produced in animals by repeated injections of *Staphylococcus aureus*. Capillary hæmorrhages are not uncommon, and are in many cases due to an increased permeability of the vessel walls, aided by changes in the blood plasma, as indicated sometimes by diminished coagulability. Similar hæmorrhages may follow the injection of some bacterial toxins, *e.g.* of diphtheria, and also of vegetable poisons, *e.g.* ricin and abrin. Skin eruptions occurring in infective diseases are in some cases due to toxins in the circulating blood, *e.g.* in scarlatina, moreover, there is the important fact that similar skin eruptions may be produced by poisoning with certain drugs. In other infections the eruption is due to the presence and activity of the organism or virus in the skin. Certain eruptions, however, may be allergic in origin. In the nervous system degenerative changes have been found in diphtheria, both in the spinal cord and in the peripheral nerves, and have been reproduced experimentally by the toxin of the diphtheria bacillus. It is probable that some of the lesions of the nervous system occurring in syphilis have likewise a toxic origin. Reactive

It is known from experimental work that such changes can be produced by

long continued toxic action a general premature ageing of the tissues also occurs. Those changes are associated with disturbances of metabolism.

B. Disturbances of Metabolism, etc. It will easily be realised that such profound tissue changes as have been detailed cannot occur without great interference with the normal bodily metabolism. General malnutrition and cachexia are of common occurrence, and it is a striking fact found by experiment that after injection of bacterial products, a marked loss of body weight often occurs which may be progressive, leading to the death of the animal. In bacterial disease assimilation is often imperfect, for the digestive glands are affected by actual poisoning with bacterial products, or by the occurrence

results in fever. The degree and course of the latter vary, sometimes conforming to a more or less definite type when the bacilli are selective in their field of operation, as in lobar pneumonia or enteric fever, sometimes being of a very irregular kind, especially when the bacteria from time to time invade fresh areas of the body, as in pyæmic conditions. The main point of interest regarding the development of fever is whether it is a direct effect of the circulation of bacterial toxins, or should be looked on as part of the reaction of the body against the invading organism. This question has still to be settled, and all that we can do is to adduce certain facts bearing on it. Thus in diphtheria and tetanus, where toxic action leading to degeneration plays such an important part, fever may be a very subsidiary feature, except in the terminal stage of the latter disease, and, in fact, in diphtheria profoundly toxic effects may be produced with little or no interference with heat regulation. On the other hand, in bacterial diseases where defensive and reparative processes predominate, fever is rarely absent, and it is nearly always present when there is an active leucocytosis. In this connection several observers have found that when a relatively small amount of the dead bodies of certain bacteria is injected into an animal, fever occurs; while the injection of a large dose is followed by subnormal temperatures and rapidly fatal collapse. Accordingly, it might appear as if the occurrence of fever had a beneficial effect and this is now supported by the results of fever therapy in certain infective diseases, *e.g.* syphilitic general paralysis of the insane, gonorrhœa. But such beneficial effect is seldom due to the bacteria being unable to multiply at the higher degrees of temperature occurring in fever. A certain amount of evidence has been brought forward to show that antibodies are more rapidly produced when the temperature of the body is above the normal, and it has been supposed that in this way fever may be of the nature of a defensive reaction. But the production of antagonistic substances may be effected without the occurrence of fever or of any apparent disturbance of health.

C. Symptoms. Many of the symptoms occurring in bacterial infections are produced by the tissue changes mentioned, as can be readily understood; whilst in the case of others, corresponding changes have not yet been discovered. Of the latter, those associated with fever, with its disturbances of metabolism and manifold affections of the various systems, are the most important. The nervous system is especially liable to be affected—convulsions, coma, paralysis, etc., being common. The symptoms due to disturbance or abolition of the functions of secretory glands also constitute an important group, forming, as they do, a striking analogy to what is found in the action of various drugs.

These tissue changes and symptoms are given only as illustrative examples, and the list might be greatly amplified. The important fact, however, is that most of the changes found throughout the organs (without the actual presence of bacteria), and also the symptoms occurring in infective diseases, either can be experimentally reproduced by the injection of bacterial poisons or have an analogy in the action of drugs.

THE TOXINS PRODUCED BY BACTERIA

Early Work on Toxins. The introduction of the principle of rendering fluid cultures bacteria-free by filtration through unglazed porcelain, and its application by Roux and Yersin to obtain, in the case of the diphtheria bacillus, a filtrate containing a toxin which reproduced the symptoms of this disease, constitute the starting-point of modern work on the subject.

Brieger had previously isolated from putrefying substances and also from bacterial cultures, nitrogen-containing bases, *ptomaines*, similar to those occurring in the ordinary metabolic processes of the body. *Ptomaines* isolated from pathogenic bacteria in no case reproduced the symptoms of the corresponding disease, and they have only historic interest.

General Facts regarding Bacterial Toxins. In dealing with the action of toxins it is necessary to distinguish between the effects produced by the actual constituents of the bacterial protoplasm (intracellular toxins, endotoxins) and those which are traceable to soluble substances in the media in which bacteria are growing (extracellular toxins, exotoxins). The former are concerned in the action of by far the greater number of pathogenic bacteria, the latter account for the pathogenic processes seen in a limited number of diseases, of which diphtheria and tetanus may be taken as outstanding examples. This distinction is of practical importance, as in consequence of these two diseases having been specially investigated early in the history of research on the subject, there was a tendency at one time to take for granted that poisons of a similar constitution are concerned in all cases of bacterial intoxication. The question also arises whether a definite distinction can be made between the two kinds of toxins, since wide variations occur in the readiness with which different organisms liberate toxic products from their bodies. It is a point of fundamental importance that substances with all the properties of toxins have only been formed by the activity of living organisms.

Exotoxins. In the cases of a few pathogenic bacteria the media in which they are growing become extremely toxic. The best examples are the organisms of diphtheria, tetanus, and botulism, in these and similar cases, when broth cultures are filtered bacterium-free toxic fluids are obtained, which on injection into animals reproduce the characteristic symptoms of the corresponding diseases. This contrasts with the pneumococcus or anthrax bacillus, filtered cultures of which are usually non-toxic. Poisons appearing in culture media have been called extracellular toxins or exotoxins. The question was debated at one time whether they are excreted by the bacteria or whether they are produced by the bacteria acting on constituents of the media. There seems little doubt now that they are diffusible products of the bacterial cell. The exotoxins are easily obtainable, but, in the past, attempts to isolate them in the pure chemical state have not been generally successful, and our knowledge of their properties was chiefly derived from the study of the toxic filtrates of broth cultures—these filtrates being usually referred to simply as the 'toxins'. Such toxins differ in their effects from the endotoxins in that specific actions on certain tissues are often manifested. Thus the toxins of the diphtheria, the tetanus, and the botulism organisms all act on the nervous system. Exotoxins of pyogenic staphylococci and streptococci produce lysis of red blood corpuscles, which may in certain instances explain the anæmia so common in the associated diseases. In the action of many of these toxins the occurrence of a period of incubation between the introduction of the poison into the animals' tissues and the appearance of symptoms is often a marked feature. Exotoxins when injected into living animals act as antigens and lead to the appearance in the blood serum of specific antibodies, these are substances which combine with the corresponding toxins and so neutralise their pathogenic action and are therefore designated 'antitoxins'.

Among the properties of the exotoxins are the following. Their quantitative potency is in many cases of an exceedingly high degree, minute amounts of culture-filtrates producing the characteristic effects. They are soluble in water and are not readily dialysable, though they vary in this respect. They

the antibodies and so preventing their action on the pneumococcus. Perhaps the most important aspect of the problem is the recognition of the existence of toxins having a direct action on the phagocytes. For example, the pyogenic cocci produce substances causing death of leucocytes and to these the term *leucocidin* has been given, but it should be recognised that such leucocidins are diffusible products and analogous to the exotoxins.

Thus, various bacterial products may enable the organisms to establish themselves in the tissues and so act as aggressins. While aggressins have been spoken of as if they constituted a separate class of toxic substances, this is very doubtful. The term describes merely bacterial products which act by interfering with phagocytosis.

Toxic Effects in Relation to Allergy. There is another point which must be kept in view, namely, that some of the phenomena which have been regarded as dependent upon the activity of bacterial toxins may possibly be related to the condition of allergy which consists in the development under certain circumstances in an animal of a supersensitiveness to foreign proteins, not toxic in themselves. Effects of the gravest kind may be produced during this period of supersensitiveness, and it has been thought that some of the phenomena of an infective disease, *e.g.* the intervention of an incubation period before symptoms occur, may be accounted for by the gradual development of supersensitiveness to the proteins of the invading bacteria; and it has been demonstrated by methods to be referred to later, that in various infections the tissues of the patient are more susceptible to the products of the invading organisms than are the tissues of a normal individual. The so-called *Shwartzman phenomenon* described in Chapter III also merits consideration in the study of bacterial lesions, and shows that the products of an organism, not primarily toxic, may so alter the reactivity of the tissues that the same products or similar substances derived from another organism may later excite an acute reaction even with hæmorrhage and necrosis. The phenomena of any bacterial disease may thus in reality be due to very different and complex causes.

The Nature of Toxins. Until recently comparatively little was known regarding this subject. The fact that many exotoxins are precipitated along with proteoses suggested that they are formed from the medium in which the bacteria are growing by processes analogous to those of enzyme action. Dernby and Walbum brought forward evidence in support of this view; thus in the case of the diphtheria bacillus they found that a marked increase of toxicity developed when an extract of the organisms or a small amount of toxin was allowed to act for some time on peptone broth. Further evidence that bacterial toxins are either proteoses or bodies having a still smaller molecule was adduced by C. J. Martin. By filling the pores of a Chamberland bougie with gelatin, he obtained what is practically a strongly supported colloid membrane through which dialysis could be made to take place under pressure, say, of compressed oxygen. In such an apparatus diphtheria toxin could pass through just as the proteoses did.

On the other hand, certain facts indicate that the exotoxins, like the endotoxins, are the product of the synthetic metabolism of bacteria. Thus diphtheria and tetanus bacilli can produce toxins when growing in protein-free fluids. Prigge found that when diphtheria bacilli were washed with a succession of changes of saline, toxin soon ceased to be extracted. At this stage treatment of the bacilli in a Buchner's press did not yield a toxic fluid. But if the washed organisms were kept under toluol for some days, then a further brief washing with saline again extracted toxin. The conclusion

drawn from such experiments was that the toxin is a constituent of the bodies of the bacilli, which is liberated after their death.

In the chemical purification of exotoxins success has been obtained recently with that of the diphtheria bacillus (Eaton; Pappenheimer; and Boivin). From cultures in a synthetic medium this toxin has been isolated in practically pure form and is a protein with a molecular weight of 15,000 to 72,000 (Pappenheimer, 1942). According to Eaton and Gronau, tetanus toxin when similarly purified fails to be precipitated by acid or alum and so differs from purified diphtheria toxin. However, tetanus toxin (tetanospasmin) has also been prepared by Pillemer *et al.* as a crystalline protein and botulinus toxin similarly by Lamanna *et al.* The fatal dose of the latter for a mouse is 0.000000033 mgm. So far, no clue has been obtained as to why these toxic proteins should exert poisonous action on higher animals. As already pointed out, not all exotoxins are destroyed by proteolytic enzymes and certain workers have obtained purified toxic products which are protein-free.

Of the nature of the endotoxins little is known. In order to produce fatal effects with endotoxins, as a rule much larger doses are required than in the case of exotoxins. They are in general much more resistant to destruction by heat than are the extracellular toxins. They are not coagulable proteins, and are not usually affected by formaldehyde, like the exotoxins. It was claimed by Macfadyen that on grinding up typhoid bacilli frozen by liquid air and allowing the material to thaw he obtained the endotoxins in liquid form, and he further stated that by using this fluid he could immunise animals not only against the toxins but also against the living bacteria. It is doubtful, however, whether antitoxins can be developed in animals by injecting endotoxins, although injections of killed bacteria, even in relatively small amounts, may give rise to agglutinating, opsonic, and bactericidal antibodies in the serum. Thus Friedberger and Moreschi found that in the human subject the intravenous injection of a fraction of a loopful of a dead culture of the typhoid bacillus gives rise to toxic symptoms, including marked febrile reaction, followed by the appearance of such antibodies.

Vaughan obtained a 'poisonous protein' by treating bacteria with alcohol and ether and then extracting them with hot alkaline alcohol (similar products were got also from blood-free tissues of higher animals and exerted poisonous action on pathogenic organisms). He concluded that generally the more highly susceptible a given animal is to infection with a given bacterium the more difficult it is to kill that animal with the cellular protein of that bacterium and vice versa. He accounted for such results by the fact that non-pathogenic organisms are destroyed by the tissues under natural conditions before they have had sufficient opportunity for proliferation to provide a dangerous dose of the poisonous protein. Vaughan's protein poisons may correspond with endotoxins. In any case,

endotoxins by the work of Boivin and Mesrobian and of Topley and Raistrick who have studied these products among various Gram-negative intestinal bacilli. Their findings show that a toxic and specific antigenic constituent can be separated from the bacterial cell which is non-protein and

consists of carbohydrate combined with phosphatide. When this compound is split by acid the carbohydrate is non-toxic and the phosphatide only slightly toxic. Such observations are of special interest in defining non-protein substances as toxins and antigens (*vide* p. 80), but they do not permit of any generalisation in regard to the chemical nature of endotoxins as a group of bacterial products.

There has been much inquiry regarding the significance of the incubation period which elapses between the introduction of many toxins into the body and the first evidence of consequent damage. This latent period is not invariably present; thus, for example, a fatal dose of staphylococcus toxin when injected intravenously into a rabbit produces its effect within a few minutes. But when tetanus toxin is injected into the substance of the spinal cord so that it comes into immediate contact with the cells on which it acts, there is an interval of some hours before the development of tetanus. It must be noted, however, that action after a latent period is not peculiar to toxins, since the harmful effects of certain physical agencies, such as an exposure to radiations, may develop after a long interval. It has been supposed in the case of toxins that they may act like ferments and initiate some change in the tissues, and that further chemical substances must then be formed before the pathogenic effects are produced. While toxins and ferments may show analogies in their liability to be destroyed by agencies such as heat, there is no close parallelism in the behaviour of the two kinds of active substance. It should be noted especially that toxins differ from ferments in that the former are strictly quantitative in their action.

Similar Vegetable and Animal Poisons. It has been found that the bacterial poisons belong to a group of toxic bodies all presenting very similar properties, other members of which occur widely in the vegetable and animal king. Examples are the ricin and abrin poisons obtained from seeds of the *Ricinus communis* and the *Abrus*. From the *Robinia pseudacacia* another poison—robin—belonging to the same group is obtained. The chemical reactions of ricin and abrin correspond to those of the bacterial exotoxins. They are soluble in water, they are precipitable by alcohol, but being less easily dialysable than the proteoses they have been called toxalbumins. Their toxicity is seriously impaired by boiling, and they also gradually become less toxic on being kept. Both are among the most active poisons known—ricin being the more powerful. When they are injected subcutaneously a period of twenty-four hours usually elapses—whatever be the dose—before symptoms set in. Both tend to produce great inflammation at the site of injection, which in the case of ricin may end in an acute necrosis, in fatal cases hæmorrhagic enteritis and nephritis may be found. Both act as irritants to mucous membranes, abrin especially being capable of setting up a most acute conjunctivitis. In the action of a poisonous fungus, *Amanita phalloides*, a somewhat similar toxin is responsible. After an incubation period of some hours, symptoms of abdominal pain, diarrhoea with bloody stools, and, later, jaundice occur. *In vitro* the toxin also has a hæmolytic action. As in the case of the toxins referred to above, neutralising antibody is present in the serum of immunised animals. This is especially noteworthy in regard to the hæmolytic principle, which is a glycoside according to Ford.

The poisons of bees, scorpions, and poisonous snakes belong to the same group. The poisons derived from the last are usually called venoms, and a very representative group of such venoms derived from different species has been studied. To speak generally, there is derivable from the natural secretions of the poison glands a series of venoms which have all the reactions of the bodies previously considered. They are not so easily dialysable as bacterial toxins, and therefore have been classed as toxalbumins. Their properties are similar to those of ricin and abrin; many of them are destroyed by heat, but the degree necessary varies much, and some will stand boiling. There is also evidence that in a crude venom there may be several poisons differently sensitive to heat. All the venoms are very powerful poisons, but there is practically no period of incubation—the effects are almost immediate. An outstanding feature of the venoms is the complexity of the crude poison secreted by any particular species of snake. C. J. Martin, in summing up the results of many observers, has pointed out that different venoms have been found to contain one or more of the following poisons: a neurotoxin acting on the respiratory

centre; a neurotoxin acting on the nerve endings in muscle, a toxin causing hæmolysis, toxins acting on other cells, *e.g.* the endothelium of blood vessels (this from its effects has been named hæmorrhagin), leucocytes, nerve-cells, a toxin causing thrombosis, a toxin having an opposite effect and preventing coagulation, a toxin neutralising the bactericidal qualities of the body fluids and thus favouring putrefaction, a toxin causing agglutination of the red blood corpuscles, a proteolytic ferment, a toxin causing systolic standstill of the excised heart. Any particular venom contains a mixture in varying proportions of such toxins; and the different effects produced by the bites of different snakes largely depend on this variability of composition. The neurotoxic, the thrombotic, and the hæmolytic toxins are very important constituents of any venom. The toxicity of different venoms varies much, and no general statement can be made with regard to the toxicity of different poisons towards man. Lamb calculated that the fatal

from general effects is given, very rapid and widespread necrosis may often occur in a few hours around the site of inoculation.

An extremely important fact was discovered by Flexner and Noguchi, namely, that the hæmolytic toxin of cobra venom in certain cases has no action by itself on red corpuscles, but produces rapid solution when some normal serum is added, the latter containing a labile complement-like body, which activates the venom. In this there is a close analogy to what holds in the case of a hæmolytic serum deprived of complement by heat at 55° C. So far no example of similar activation of a bacterial toxin is known, but the results mentioned point to the possibility of this occurring in some cases in the tissues of the body.

There is another group of toxic manifestations which present some analogies to those of the bacterial toxins, the best example of these is found in the toxic properties of the serum of the eel. If a small quantity of such serum, say 0.25 c.c., be injected into a rabbit subcutaneously, death occurs in a few minutes. Nothing is known of the substances giving rise to such effects.

all possess the further property that, as will be afterwards described, when introduced into the bodies of suitable animals they act as *antigens* and stimulate the production of neutralising substances called *antitoxins*. The nature of the antagonism between toxin and antitoxin will be discussed later. Here, to explain what follows, it may be stated that (1) the toxin forms directly a combination with the antitoxin, and (2) it may lose much of its toxic power (as a result of keeping, or through treatment with certain chemicals, *e.g.* formaldehyde) and still be capable of uniting with exactly the same proportion of antitoxin. From these and other circumstances Ehrlich advanced the view that the toxin molecule has a complicated structure, and contains two atom groups. One of these, the *haptophore* (*ἁπτειν*, to bind to), is that by which combination takes place with the

occurs in, say, diphtheria toxin, on the theory that the *toxophore* group undergoes disintegration. If we suppose that the *haptophore* group remains unaffected, we can then understand how a toxin may have its toxicity diminished and still require the same proportion of antitoxin molecules for its neutralisation. To the bodies whose *toxophore* atom groups have become altered in this way, Ehrlich gave the name *toxoids*. His view with regard to the origin of toxoids from toxins has received ample confirmation within recent years by the work of Ramon and others, and is now generally accepted.

The theory may afford an explanation of what has been suspected, namely, that in some instances toxins derived from different sources may be related to one another. For example, Ehrlich pointed out that ricin produces in a susceptible animal an antitoxin which corresponds almost completely with that produced by another vegetable poison, robin (*vide supra*). This may be explained on the supposition that the haptophore groups of ricin and robin correspond, while their toxophore groups differ. The whole subject will be referred to again in the chapter on Immunity.

CHAPTER III

IMMUNITY

By immunity is meant non-susceptibility to infection by a pathogenic organism or to its toxins. Immunity may be possessed by an individual naturally, and is then usually called *natural* immunity, or it may be *acquired* by passing through an attack of the disease, or the same result may be obtained by means of artificial inoculation. It is to be noted that man and the lower animals may be exempt from certain diseases under natural conditions, and yet the causal organisms of these diseases may produce pathogenic effects when injected in sufficient quantity. Immunity is, in fact, of very varying degrees, and accordingly the term is used with a relative significance—this is not only true of infection by bacteria, but of toxins also. When the resistance of an animal to a toxin is of high degree, it may nevertheless be overcome by a very large dose. On the other hand, even in cases where the natural resistance is high, this can be still further exalted by artificial means—that is, the immunity may be artificially intensified. It is not yet clear whether acquired immunity depends simply on an exaggeration of the mechanisms of natural immunity or whether it is essentially different.

Acquired Immunity in the Human Subject. The following facts are supplied by a study of the infective diseases which affect the human subject naturally. First, in the case of certain of those, one attack protects against another for many years, sometimes practically for a lifetime, *e.g.* smallpox, typhoid, scarlet fever, etc. The protection is specific, since the person remains susceptible to other infections. Secondly, in the case of other diseases, *e.g.* erysipelas, influenza, gonorrhœa, etc., a patient may suffer from more than one attack. In the case of the diseases of the second group, however, experimental research has shown that in many of them immunity is developed, but is transient, and, though we cannot definitely state it as a universal law, it must be considered highly probable that the passing through an attack confers immunity for a longer or shorter period. The immunity is not, however, to be regarded as the result of the disease *per se*, but of the reaction to bacterial products formed in the body during the disease. It has

process of immunisation would appear to be going on during the progress of the disease, and when this immunisation has reached a certain height, the disease naturally comes to an end. The immune substances are found in the plasma and the serum, the term '*antibodies*' has been applied to them. Such antibodies represent a biological reaction to certain foreign substances, called *antigens*, which have come into contact with the host's tissues, the immunity reaction is really independent of the occurrence of symptoms of disease. It should be noted that different individuals, even of the same species, may vary enormously in the degree of their antibody response to a given dose of antigen. While the changes undergone by the plasma and serum of immunised individuals are as a rule readily demonstrable, it must be understood that it is difficult to detect alterations in the tissue cells which

constitute the source of the antibodies. In many cases also phagocytic cells are instrumental in finally destroying pathogenic organisms which have been acted on by antibodies ; accordingly, a state of immunity frequently results from the combined action of antibodies (the 'humoral' factor) along with tissue cells. The study of the latter belongs to pathology ; here the chief concern is with properties of the plasma or serum of immunised individuals—serology.

Another principle is exemplified by vaccination against smallpox (variola), namely, that immunity may be conferred as the result of passing through a *modified* attack of a disease. Vaccinia virus is derived from that of smallpox by passage through the cow. As a result it becomes altered, so that when vaccination is performed the consequent pathogenic effects are slight as compared with those of unmodified smallpox. Although the degree of immunity conferred by vaccination is less complete and lasts a shorter time than that produced by a natural attack of variola, nevertheless a very effective protection is produced against the serious effects of the latter. It will be seen, as a further development of this principle, that by suitable gradation of the doses of bacteria or their products, or by the use of attenuated bacteria or weakened toxins, a high degree of immunity may be attained without the occurrence of any symptoms whatever.

What has been stated above with regard to infections of man applies generally to those of other mammals.

ARTIFICIAL IMMUNITY

According to the means by which it is produced, immunity may be said to be of two kinds, to which the terms *active* and *passive* are generally applied.

Active immunity is obtained by introducing into the tissues, *i.e.* 'parenterally', (a) the causal organisms of a disease either in an attenuated condition or in sub-lethal doses, or (b) sub-lethal doses of their products, *i.e.* of their 'toxins', the word being used in the widest sense. By repeated injections at suitable intervals the dose of organisms or of the products can be gradually increased, or, what practically amounts to the same, an organism of greater virulence or a toxin of greater strength may be used. The establishment of immunity is commonly attended by the appearance of antibodies in the serum, and the constituents of the bacteria or toxins which lead to the development of these are antigens. Such methods constitute the means of *preventive inoculation* or *vaccination*. Immunity of this kind is comparatively slowly produced and lasts a considerable time, the duration varying in different cases.

Passive immunity depends upon the fact that if an animal be immunised to a high degree by the active method, its serum may have considerable antagonistic or neutralising effects when injected into another animal along with the organisms or with their products, as the case may be ; that is, the antibodies developed by active immunisation may be transferred to a fresh animal. Such a serum, generally known as an *antisera*, may exert its effects if introduced into an animal at the same time as infection occurs or even a short time afterwards ; it can, therefore, be employed as a *curative agent*. The serum is also *preventive*, *i.e.* protects an animal from subsequent infection, but the passive immunity thus conferred lasts a comparatively short time. These facts form the basis of *serum therapy*. When such a serum has the power of neutralising a toxin it is called *antitoxic* ; when, with little or no antitoxic power, it protects against the living bacterium in a virulent condition, it is called *antimicrobial* or *antibacterial* (*vide infra*).

Immunity towards the various classes of infective agents—bacteria, spirochaetes, protozoa, fungi, filter-passing viruses, etc.—appears to depend upon the same principles. Further, the differences which may be presented in the case of different members of any one of these groups are at least as pronounced as those observed between the different groups as a whole. Active immunity of high degree developed by the methods described may be regarded as specific in the sense explained later.

In the accompanying table a sketch of the chief methods by which immunity may be artificially produced is given. It has been arranged merely for purposes of convenience and to aid subsequent description, the principles underlying all the methods are the same.

ARTIFICIAL IMMUNITY—METHODS OF PRODUCTION

A ACTIVE IMMUNITY, *i.e.* produced in an animal by the introduction, once or repeatedly, of non-lethal doses of an organism or its toxins. It is essential, as a general rule, that the antigen should be *injected into the tissues*, that is, *parenterally*, and not introduced into the alimentary tract.

1 *By living organisms*

(a) Attenuated in various ways. Examples

(1) By growing in artificial culture media. (2) By growing at abnormal temperatures, etc. (3) By growing in the presence of weak antiseptics, or by injecting the latter along with the organism, etc. (4) By passing through the tissues of an animal of different species from the one to be immunised.

(b) In a virulent condition, in non-lethal doses.

(a) and (b) may be followed by the introduction of organisms of exalted virulence.

2. *By dead organisms*

3 *By filtered bacterial cultures, i.e. toxins, or by substances derived from such filtrates, i.e. toxoids (anatoxins)*

These methods may also be combined in various ways.

B PASSIVE IMMUNITY, *i.e.* produced in one animal by injection of the serum of another animal highly immunised by the methods of A.

1 *By antibacterial serum, i.e.* the serum of an animal highly immunised against a particular bacterium in the living and virulent condition.

2 *By antitoxic serum, i.e.* the serum of an animal highly immunised against a particular toxin.

METHODS OF PRODUCING ACTIVE ARTIFICIAL IMMUNITY

1. **By Living Cultures.** (a) ATTENUATED. In the earlier work on immunity to anthrax, fowl cholera, swine plague, etc., the investigators had to deal with organisms of high virulence, accordingly the virulence had to be reduced before the organisms could be injected in the living state. It is now found most convenient as a rule to start the process of active immunisation with the injection of dead cultures. The principle is the same as that of vaccination, and both attenuated cultures and also the dead cultures used for injection are often spoken of as *vaccines*. It is doubtful whether

(Metchnikoff and Besredka), etc., living organisms are more effective than dead organisms as a vaccine. The same is true of many of the filter-passing viruses.

Attenuation of the virulence of organisms. The virulence of an organism may be diminished in various ways, of which the following examples may be given.

(1) In the first place, most organisms when cultivated for some time outside the body, lose their virulence to a greater or less degree, and in the case of some this is very marked indeed, *e.g.* the pneumococcus. Pasteur found in the case of fowl cholera, that when cultures were kept for a time in ordinary conditions, they gradually lost their virulence, and that when subcultures were made the diminished virulence persisted. Such attenuated cultures could be used for protective inoculation. He considered the loss of virulence to be due to the action of the oxygen of the air, as he found that in tubes sealed in the absence of oxygen the virulence was not lost. Haffkine attenuated cultures of the cholera vibrio by growing them in a current of air.

(2) Many organisms become diminished in virulence when grown at an abnormally high temperature. The method of Pasteur, for producing immunity in sheep against anthrax bacilli, depends upon this fact.

(3) Still another method may be mentioned, namely, the attenuation of the virulence by growing the organism in the presence of weak antiseptics. Chamberland and Roux, for example, succeeded in attenuating the anthrax bacillus by growing it in a medium containing carbolic acid in a concentration of 1/600. In Calmette and Guérin's method of immunising against tuberculosis a culture of the tubercle bacillus is used which has become highly attenuated as a result of prolonged growth on a medium containing bile.

(4) The virulence of an organism for a particular animal species may be lessened by passing the organism through another species. Duguid and Burdon Sanderson found that the virulence of the anthrax bacillus for bovine animals was lessened by passage through guinea-pigs, the disease produced in the ox by inoculation from the guinea-pig being a non-fatal one. A similar principle was applied in the case of swine erysipelas by Pasteur, who found that if the organism producing this disease was inoculated from rabbit to rabbit, its virulence was diminished for pigs. The method of vaccination against smallpox depends upon the same principle.

These examples serve to show the principles underlying attenuation of the virulence of an organism. There are, however, still other methods, most of which consist in growing the organism in relatively unfavourable conditions.

Depression immunity. A striking phenomenon in active immunisation was described by Morgenroth *et al.* They found that when mice were the subjects of a chronic general streptococcal infection, the effects of an additional injection of virulent streptococci were diminished; and the important point is that such mitigation was manifest within twenty-four hours after the original inoculation.

ing or depressing
—hence the term
further elucidation.

(b) **LIVING VIRULENT CULTURES IN NON-LETHAL DOSES.** Immunity may also be produced by employing virulent cultures in small, that is, non-lethal, doses. This may be effected by injecting the organisms into a situation which is unfavourable for the development of the pathogenic action, *e.g.* the intravenous injection of the *Bacillus chauvæi*. In subsequent inoculations the doses may be increased in amount. Such a method, however, is difficult to control, and it has generally been found more convenient to commence the

process of immunisation with dead or attenuated cultures, and then to continue with virulent cultures

Exaltation of virulence of organisms. The converse process to attenuation, *i.e.* the exaltation of the virulence, is obtained chiefly by the method of rapidly transferring the organism from animal to animal of the same species—the method of *passage*—which was discovered by Pasteur (first, it appears, in the case of an organism obtained from the saliva in hydrophobia, though having no causal relationship to that disease) This is conveniently done by injection into the peritoneum or circulation. When the animal dies or is killed the organisms in the heart blood or peritoneal fluid may be used for the subsequent injection, or a culture may be made between each inoculation. The virulence of many organisms can be increased in this way, the animals most frequently used being rabbits and guinea-pigs. This method can be applied generally to those organisms which invade tissues, pneumococci, streptococci, and staphylococci, etc., as well as to those of typhoid and cholera. It is known that cultures of a given species of organisms may vary in virulence, and this is sometimes associated with differences in colony appearance (p. 26). Within the animal body there may be a selective multiplication of virulent strains, although there is also evidence that a true enhancement of virulence in response to environment occurs, in some instances this is indicated by the formation of a capsule. In many instances however, it is difficult or even impossible to exalt the virulence of an organism by passage. Also, several strains of an organism may show differences in virulence which so far cannot be correlated with differences in other properties already referred to. As has been mentioned, a decapsulate variant may be rendered virulent by treatment *in vitro* which produces a capsule again. It is noteworthy, however, that this procedure is not effective unless the variant strain was derived originally from a virulent organism (Shaffer, Enders *et al.*).

2. By Dead Cultures. In some cases a high degree of immunity against infection by a given organism may be developed by repeated and gradually increasing doses of killed cultures, the method of killing being sometimes by heat, sometimes by exposure to some antiseptic, *e.g.* formalin, etc. In this procedure the so-called endotoxins will be injected along with the other substances in the bacterial protoplasm, but the resulting immunity is chiefly directed against the vital activity of the organisms, *i.e.* is antibacterial rather than antitoxic (*vide infra*). It is, however, not universally applicable. Dead

cultures; this method is extensively used for experimental purposes. The use of dead cultures is adopted in man in anti-typhoid and anti-plague inoculations, etc., and also for the treatment of infections by means of vaccines. When killed cultures are to be used for purposes of immunisation it is important to ensure that the organisms possess the most efficient antigens for developing protective antibodies. As has been seen, variants tend to develop in cultures on artificial media; for example, when the normal type of the colony is 'smooth', 'rough' forms appear which lack the capacity to produce a solid immunity against the respective infection.

By sensitised dead cultures. In this method, which was originally introduced by

Afterwards the doses are gradually increased. This method is

being produced.

Active Immunity by Feeding. Ehrlich found that mice could be gradually immunised against ricin and abrin by feeding them with increasing quantities of these substances (*vide* p 68). In the course of some weeks' treatment in this way the resulting immunity was of so high a degree that the animals could tolerate on subcutaneous inoculation 400 times the dose originally fatal. Fraser also found in the case of snake venom that rabbits, by being fed with the poison, could be immunised against several times the lethal dose of venom injected into the tissues. In such cases some of the constituents which act as antigens apparently pass through the intestinal wall unchanged. Vaccines have also been administered by mouth in the case of typhoid and Shiga's dysentery bacillus, but the immunity produced seems to be less than after parenteral administration. The method is of restricted application.

Practical Applications. The following may be mentioned as some of the most important practical applications of the principles of active immunisation, with a view to the prevention of disease, *i.e.* of protective inoculation.

(1) Vaccination of sheep and cattle against anthrax (Pasteur); (2) Jennerian vaccination against smallpox; (3) Anticholera vaccination (Haffkine); (4) Anti-plague vaccination (Haffkine); (5) Anti-typhoid vaccination (Wright and Semple); (6) Pasteur's method of inoculation against hydrophobia, which involves essentially the same principles; (7) Toxin-antitoxin or toxoid (anatoxin) immunisation against diphtheria and tetanus; (8) Vaccination against Rickettsia infections—Rocky Mountain spotted fever and the typhus fevers; (9) Vaccination against dog distemper; (10) Vaccination against yellow fever.

In order to maintain active immunity to the above diseases at an effective level it is usually necessary to repeat immunisation at intervals of one to several years according to the particular infection.

Local Immunisation. A certain degree of immunity, or rather of increased general resistance of parts of the body (for example, the peritoneum), can be produced by the injection of various substances—broth, blood serum,

According to Besredka, killed cultures of streptococci do not confer immunity when injected subcutaneously, but do so when injected into the skin, the larger the number of points of injection the higher being the immunity. Filtrates of cultures are also effective, they contain 'antivirus' which produces immunity only when injected into the skin or applied over the surface. A somewhat similar example of local immunity was observed in the case of infection through the skin in anthrax. He finds also that it is possible to produce a local immunity of the respiratory tract against *B. diphtheriae* by intratracheal injection of the organism in the dead condition; and of the intestine against *B. paratyphosus* by previous oral administration of ox bile with the killed

instead of antivirus; again, antibodies are not invariably absent from the serum of animals immunised by Besredka's methods. Accordingly the insusceptibility to infection which is developed by means of Besredka's procedures would appear to depend partly on local changes involving the reticulo-endothelial system (p 58), which are non-specific in character, and partly on the production of active immunity in response to the specific antigens introduced.

ANTIGENS AND THE PROPERTIES OF THE SERA OF ACTIVELY IMMUNISED ANIMALS (ANTIBODIES)

The fundamental fact in passive immunity, namely, that immunity can be transferred by injecting the serum from an actively immunised animal into a susceptible one, shows that the serum in question differs from the serum of a normal animal in containing antagonistic substances to the bacterium or toxin as the case may be—these being generally spoken of as antibodies. There is considerable individual variation in capacity to produce antibodies; young animals tend to react weakly. The development of these antibodies, first observed in the case of toxins, is found to occur when a great many different substances are introduced into the tissues of the living body. In fact, organic compounds can be divided into two classes, namely, those which give rise to the production of antibodies, and are thus known as antigens, and those which do not possess this property. It will be seen that antibodies combine with the corresponding antigens; as a result certain effects follow, which may be manifested either in the living animal or *in vitro*.

Antigens. Among the antigens originally studied are various toxins, tissue cells, bacteria, red corpuscles, soluble tissue constituents of animal or vegetable origin, etc. They are complex organic products and all probably contain a protein constituent, though their true composition is not known, and none of them has been obtained in a pure condition. Proteins in process of hydrolysis rapidly lose their antigenic function, amino-acids and probably also peptones being devoid of the property; the same is true of proteins racemised by treatment with alkali. All native proteins, however, are not equally potent as antigens; denaturation by heat tends to impair the antigenic property, an antiserum to the denaturated protein reacts with the latter, producing precipitation, but scarcely precipitates the native protein. Among the substances which do not act as antigens are the various poisons of known constitution, glycosides, alkaloids, etc., by themselves. It may be stated generally that substances of non-protein nature seldom act as antigens. However, more recent investigations on constituents extracted from natural antigens has shown that various lipoids and polysaccharides of high molecular weight and especially complexes of such compounds, can act as antigens. Again, some substances of those classes, although lacking the capacity to develop antibodies *in vivo*, may be capable of combining *in vitro* with antibodies developed by the complex antigens from which they were obtained. Such reactive components are termed *haptens* (p. 80). Haptens may be converted into true antigens by injecting them into an animal as a mixture with a foreign protein. The physical state of an antigen can affect its capacity to evoke antibodies, thus adsorption on particles of collodion, kaolin, etc., precipitation with alum, or even addition of dead bacteria may enhance antigenic power. In this way apparently bacteria coated with agar may on injection develop antibodies to the latter substance as well as to the bacterial antigens proper. On the other hand, when several antigens reach the tissues simultaneously, one may suppress the antibody response to another ('competition of antigens').

The antibody forms a chemical or physical union with the particular antigen which has led to its development, and the evidence for this will be discussed later. Furthermore, the antibody has apparently a specific combining group which, as it were, fits a group in the corresponding antigen in a manner comparable to that of a lock and key. It is, however, to be noted that this specificity is chemical or physico-chemical rather than biological.

For example, an antiserum developed by the injection of bacterium A may also have some effect on a related bacterium B, and thus appear not to be specific. However, it can be shown that the antigens in bacterium A are not all identical (they are sometimes referred to as a 'mosaic'), and that some of them are present, though in smaller proportion, in bacterium B, thus the theory of combining specificity is not invalidated. It is noteworthy in this connection that spores are antigenically distinct from the vegetative forms of the homologous bacilli. The number of different antibodies, as judged by their combining properties, would appear to be almost unlimited, a fact which illustrates in a striking way the complexity of the structure of living matter.

Specificity The investigation of the properties of animal proteins as antigens has shown that in general antibodies are developed only when the material is injected into an animal of another species, i.e. the character of foreignness is essential. Also, the proteins of, say, the serum of one animal species can be shown to differ from those of other species by the antibodies which they evoke. But certain proteins may lack this species specificity, e.g. of the lens of the eye; in addition, the study of human blood groups and experimental work with animals' blood have demonstrated that the red blood corpuscles of all individuals of the same species are not identical antigenically. Further, specific differences have been found in the antigens of different organs of the same individual. With some species of bacteria a number of

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Pick and of Landsteiner on modification of the antigenic function of protein by chemical means is of great importance. This has been effected by introducing new groups such as iodine, azo- and nitro-radicals, by acetylation or methylation of proteins, also treatment with mustard gas, phenyl isocyanate, etc. Such modified proteins act as specific antigens and each tends to give rise to a corresponding antibody even in the animal from which the protein was originally derived. For example, Landsteiner and Jablons found that rabbits injected with acetylated horse serum develop antibodies which react specifically with acetylated serum-protein from various other animals (hen, rabbit) in addition to the horse, but not with normal horse's serum or with diazo- or nitro-serum protein. Rabbits also produce an antiserum to acetylated rabbit serum. The serum has thus been deprived of its original species specificity and a new specificity has been acquired, which may be called *structure specificity*. Again, with antigens consisting of protein chemically coupled with various diazotised aromatic organic compounds, it was found that an antiserum developed in the rabbit by arsanic acid azo-horse serum protein reacted *in vitro* with chicken serum or egg-albumin compounds containing the arsenical group, but not with similar compounds containing other acid groups. Thus it was proved that the arsanic acid, although a comparatively simple substance, had conferred specificity on the antigen. Therefore structure specificity depends on the chemical constitution of the 'determinant group' or compound which has been combined with the serum proteins. This specificity is so delicate that an antiserum to a compound of l-tartaric acid with protein shows little or no reaction with the corresponding antigen derived from d-tartaric acid (Landsteiner and van der Scheer). Similarly the α and β forms of glucose can be distinguished (Avery *et al*). Such results have an important bearing on views regarding the mode of formation of antibodies under the influence of antigens, as will be discussed later.

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Similarly the α and β forms of glucose can be distinguished (Avery *et al*). Such results have an important bearing on views regarding the mode of formation of antibodies under the influence of antigens, as will be discussed later.

Haptens. The specific relations of antibodies to antigens can be shown by certain reactions *in vitro* (*vide infra*). As has been stated already, the term antigen should be restricted to a substance which leads to the development of an antibody; there are, however, substances which do not possess the antigenic function, but which may still give specific reactions *in vitro*. Such substances are usually known by the name of haptens, given to them by Landsteiner. He showed that in the case of antisera developed against proteins combined with various substances of known constitution (e.g. organic amines, acids, etc.), such substances might display a specific combination with the antibodies developed against the protein-complexes, though when introduced into the body the non-protein substances did not by themselves act as antigens. Again, in the case of the Forssman hæmolytic antibodies (p. 97) it was found that the lipoidal fraction of the antigen reacted with the corresponding antibody *in vitro*, although on injection into animals it did not lead to the development of antibody. Another example of non-protein haptens is given by the group of bacterial polysaccharides or specific soluble substances to which certain antibody reactions are due. Research on this subject, dating from the work of Dochez and Avery (1917) on the pneumococcus, has shown that such carbohydrate compounds are present in various other bacteria, and that it is to them that the specific characters of antisera to different species or types within a species are due. Thus the reactions by which different types of pneumococci are distinguished have been shown to be due to combinations of such polysaccharides with bacterial protein acting as antigens, while antibodies to the pneumococcus as a species are developed by its protein antigen alone. The compound of polysaccharide and protein resides especially in the capsules of the bacteria, and as capsule formation is often associated with virulence, virulent strains are specially effective in leading to the development of type-specific antibodies. By injecting into rabbits an artificial antigen consisting of a chemically defined compound, the amino-benzyl glycoside of cellobiuronic acid, diazotised and coupled with the globulin of normal horse serum, an antibody has been obtained which *in vitro* causes capsule-swelling and agglutination of type III pneumococcus, and precipitation with the capsular polysaccharide; this antibody also protects mice against infection with virulent pneumococci of types II, III, and VIII. Further, a chicken serum cellobiuronic acid antigen yields precipitate with antiserum developed in horses or rabbits by the above types. A similar artificial antigen containing glycuronic acid, an isomer of cellobiuronic acid, develops an antiserum which protects mice against virulent type II pneumococci, but not against types III or VIII. Thus clearly there is overlapping between the antigenic properties of these polysaccharides and those occurring naturally in the capsules of the several pneumococcus types. Accordingly, certain substances—carbohydrates, lipoids, organic acids, etc. (all non-antigenic in themselves)—when combined with protein confer on the latter a specific antigenic property, and may by themselves give a specific reaction with the corresponding antibody *in vitro*. On the other hand, protein is as a rule essential for evoking the antigenic function.

Non-protein antigens. Until recently it was believed that the bacterial polysaccharides were quite devoid of antigenic action, but Heidelberger and Goebel have found that if the specific soluble substance of type I pneumococcus is extracted from the organisms by measures which avoid treatment with alkali, some degree of antigenic property is retained. The action of alkali on this substance, however, causes the splitting off of an acetyl group and leads to a product which is non-antigenic, although possessing hapten properties. Gram-negative bacilli of the salmonella group, e.g. *B. aertrycke*,

in their smooth form yield a complex antigenic substance which contains polysaccharides along with lipoids (compounds of acetic acid, fatty acids, and phosphoric acid). There are several methods for obtaining the antigen from the organisms—extraction with N/4 trichloroacetic acid in the cold (Bovin *et al.*); digestion of acetone-killed organisms with trypsin and precipitation from the soluble portion with alcohol—50 to 68 per cent. by weight—(Raistrick and Topley); or extraction with diethylacetone (Morgan). The antigens are large molecules

tions and on injection into

(granular) agglutination of the homologous organisms. Also antisera protect mice against infection with the virulent culture. The substances are toxic and this property is neutralised by the antisera, although not powerfully, hence Bovin considers that the polysaccharide-lipoid complex as well as being the somatic antigen, also represents the chief part of the endotoxin of the organisms; rough forms usually lack it. *B. typhosus* possesses a corresponding antigen; and the Vi antigen is of similar nature, although differing in chemical constitution and in being precipitated by uranium salts. The separated polysaccharide-lipoid antigens in neutral solution resist heating to 100° C.; in weak acid solution they are split up. A number of other Gram-negative organisms possess such antigens, e.g. Shiga's and Flexner's dysentery bacilli, the cholera vibrio, the *Bacillus proteus*, meningococcus,

glycol contains as an essential constituent a polypeptide-like substance.

ACTION OF ANTIBODIES

When antibodies react *in vivo* or *in vitro* with the corresponding antigens (or haptens), it is found that (a) in certain cases simple combination may occur (e.g. antitoxic action), (b) in other cases physical effects may be associated with combination (e.g. agglutination or precipitation) and (c) in a third group of cases the antibody may lead to the union of another body normally present in serum, called complement or alexin. In this third group the combination of complement may or may not result in physical change in the antigen, the evidence of the latter occurrence being elicited by the complement-fixation reaction. Antibodies of the third class are known as immune-bodies or amboceptors (Ehrlich) or sensitising substances—*substances sensibilisatrices* of French writers.

Such are the three classes of antibodies usually recognised, but while the classification is convenient they must not be regarded as necessarily distinct. For instance the combination of toxin and antitoxin may be attended by a physical change, namely, flocculation, and it is also known that the antibody which acts as a precipitin is closely related to, if not identical with, the antibody which leads to the fixation of complement. It must be added, however, that the nature of the antigen, as well as the route by which it is injected (subcutaneous, intravenous, etc.), are important factors in determining the characters of the antibody formed (Treffers *et al.*)

As has been seen, the development of immunity is commonly associated with the appearance of antibodies in the blood. In the case, especially, of non-poisonous antigens, e.g. certain killed bacteria or foreign blood cells, which are effective when a single dose is injected, the curve of antibody production can be studied by withdrawing specimens of blood at intervals and

examining the serum. It is found that after several days antibodies begin to appear and they increase almost critically after five to seven days and reach their height about ten days after the injection. Thereafter they gradually decline, but can be still further increased by a subsequent larger dose. When the stimulus of fresh doses of antigen ceases, the production of antibodies finally comes to an end. But animals which have been immunised for a long time and have responded by a high production of antibodies, *e.g.* antitoxins, may continue to form these almost indefinitely when immunisation ceases (Barr and Glenn). It is to be noted, however, that the presence of antibodies in the blood is not essential to immunity, since after they have disappeared from the blood the animal may still possess immunity. There is thus some change effected in the cells of the body which results in protection, but its nature is not fully known. It has been found, however, that an immunised animal without antibodies in the blood reacts in a different way from the normal animal, inasmuch as antibodies are formed more rapidly and more abundantly on reintroduction of the antigen. But whether this altered power of antibody production is the full explanation of the immunity, is still doubtful. It is certain that in some cases the immunity resides locally in the tissues, *e.g.* in the skin when immunity to erysipelas has been established.

As met with in untreated specimens of antisera, antibodies are damaged or destroyed by agencies which denature proteins. Thus alcohol at room-temperature is highly destructive, but much less so below 0°C . While they may resist heating at 55° to 65°C for several hours, higher temperatures are rapidly destructive, also, when antisera are kept at 16° to 37°C . for long periods antibodies gradually disappear. It has been found recently by Kleczkowski that in the process of heating antisera (from rabbits) short of destruction of antibodies by denaturation, other changes occur. Complexes are formed by the antibodies with non-specific protein constituents. Such complexes may combine with the corresponding antigen, but fail to bring about the usual reaction, *e.g.* agglutination or precipitation. The degree of interference depends on the particular antigen. Thus it is large with the somatic antigen of bacteria but small with the flagellar. On the other hand, desiccation or freezing preserves antibodies practically indefinitely. The gradual deterioration of antibodies when sera are stored under ordinary conditions is of practical importance in connection with their therapeutic use. The theoretical aspects will be taken up again later after considering the actual properties of antitoxic and of antibacterial sera.

(1) ANTITOXIC ACTION

A distinction has been drawn between exo- and endo-toxins (p 63). With regard to these the general statement may be made that while antitoxins are, as a rule, comparatively easily obtained to exotoxins, this is not the case for endotoxins. It is an important fact that by the injection of dead cultures an active antibacterial serum can frequently be developed which, however, has no neutralising action on the endotoxins, and we must conclude that many endotoxins are not antitoxinogenic (p 64). The best examples of antitoxic sera are those to the toxins of diphtheria, tetanus, and botulism, as well as to ricin and abrin, and to snake poisons. Here diphtheria antitoxin will be referred to chiefly, but the principles and methods described apply to antitoxins generally, although some modifications are required in the case of other antitoxins, *e.g.* for the estimation of the immunity unit, the species of animal used, etc. The steps in the preparation of antitoxin are the following:

first, the preparation of a powerful toxin; second, the estimation of the power of the toxin; third, the development of antitoxin in the blood of a suitable animal by gradually increasing doses of the toxin, fourth, the estimation from time to time of the antitoxic power of the serum of the animal thus treated. For therapeutic use antitoxic serum of high potency is required. In order to secure this, two methods are followed: (1) the process of immunisation is pushed, and (2) the antiserum is treated so as to separate those protein fractions which possess antitoxic action and obtain them in concentrated solution.

1. *Preparation of the toxin* The organisms are grown in a suitable fluid medium and when the maximum degree of toxicity has been reached the bacillary bodies are removed. The term 'toxin' is usually applied for convenience to the bacterium-free product. The general properties of toxins have been dealt with already; the mode of preparation and the conditions affecting the development of diphtheria and tetanus toxins are described later.

2. *Estimation of the toxin* The power of the toxin is estimated by the subcutaneous injection of varying amounts in a number of guinea-pigs, and the minimum dose which will produce death is thus obtained. This, of course, varies in proportion to the weight of the animal, and is expressed accordingly. In the case of diphtheria toxin, in Ehrlich's standard, the minimum lethal dose—known as M.L.D.—is the smallest amount which will cause death in a guinea-pig of 250 (230 to 280) grms. within five days. This direct method of testing a toxin is a tedious process, because the toxicity diminishes with time. Accordingly it is found more convenient to test toxins by finding how much will be neutralised by a certain amount of a standard antitoxic serum, namely, an 'immunity unit' (p. 84).

3. *Development of antitoxin.* At first small animals, such as guinea-pigs, were used for experiments on the development of diphtheria and tetanus antitoxins, afterwards sheep and goats, and finally horses were employed as a source of therapeutic antisera. In the case of small animals it was found advisable to use in the first stages of the process either a weak toxin or a powerful toxin modified by certain methods. Such methods are the addition to the toxin of trichloride of iodine (Behring and Kitasato) or Gram's iodine solution (Roux and Vaillard), and the plan adopted by Vaillard in the case of tetanus, of using a series of toxins weakened to varying degrees by exposure to different temperatures, namely, 60°, 55°, and 50° C. In recent years formalin (0.3 to 0.4 per cent) has been used by Ramon, Glenny and others, in the early stages of immunisation. By leading to the formation of toxoid (p. 69)—called by Ramon 'anatoxin'—it diminishes the toxicity of the toxin without appreciably affecting its antigenic property. The nature of the change undergone by toxin in conversion to toxoid is still obscure, however. In the case of large animals immunisation is sometimes started with small doses of unaltered toxin, and the doses are gradually increased. It has long been recognised that horses vary greatly in their resistance to diphtheria toxin, and also that some of them have a certain amount of antitoxin in their blood under natural conditions. Glenny has found that such animals produce antitoxin rapidly, whilst those with no natural immunity do not respond readily to immunisation. The production of diphtheria antitoxin occurs most satisfactorily when the toxin is injected subcutaneously or intramuscularly, when the intravenous route is used in the later stages antitoxin formation is deficient or may be almost nil (Dean). The antitoxin content of the serum is estimated from time to time, the object of immunisation being, of course, to raise it to as high a level as possible. It is found that each injection produces at first a certain amount of fall in the antitoxin value—the so-called

'negative phase'—which is only in part due to the neutralising action of the toxin injected; and this, in favourable cases, is followed by a rise to a higher level than before. (Similar phenomena are observed in the development of all other classes of antibodies.) In all cases of immunising, the general health of the animal ought not to suffer. If the process is pushed too rapidly the antitoxic power of the serum may diminish instead of increasing, and a condition of marasmus may set in and may even lead to the death of the animal. The response to the first injection of toxin is slight and occurs slowly; but if a considerable interval is allowed to elapse and then a second dose is given, antitoxin is formed more rapidly and in greater amount than would have occurred had the same dose been given to a normal animal. As Glenny and his co-workers have expressed it, this 'secondary stimulus' in an animal which has already developed basal immunity is much more effective than the 'primary stimulus'; advantage is taken of this fact in spacing the injections of toxin (or toxoid) so as to obtain the best immunity response. Also, as found by Barr and Glenny, the secondary response may be followed several months later by a further 'delayed response', although there has been no additional injection of antigen in the interval. The degree of the response depends too on a variety of other factors which have not been completely defined. Non-specific factors such as the addition to the toxin of substances which delay its absorption, e.g. tapioca, alum, or lanoline may intensify the response. On the other hand, constituents of the medium in which the toxin has developed may interfere with the immunity response. After a sufficiently high degree of antitoxic power has been reached, the animal is bled under aseptic precautions, and the serum is allowed to separate in the usual manner. It is then ready for use, but some antiseptic, such as 0.5 per cent tricresol, is usually added as a preservative. Other antitoxic sera are prepared in a corresponding manner. Further facts about antidiphtheria and antitetanus serum are given under the respective organisms.

4. *Estimating the antitoxic power of the serum.* This is done by testing the effect of various quantities of the serum of the immunised animal against a certain amount of toxin. Various standards have been tried, of which Ehrlich's is that now chiefly used. Ehrlich adopted originally as the *immunity unit* the amount of antitoxic serum which neutralised 100 times the minimum lethal dose of a particular specimen of toxin—serum and toxin being mixed together, diluted up to 4 c.c., and, after standing for a short time, injected subcutaneously into a guinea-pig of 250 grms. weight; the prevention of the death of the animal, together with absence of symptoms or of œdema at the site of injection was taken as the indication of neutralisation. However, this evidence of complete neutralisation of toxin by antitoxin was found difficult to assess in practice. Accordingly, the amount of toxin in the mixtures was subsequently increased, the smallest quantity being used which when mixed with one immunity unit of serum and injected into a guinea-pig as above described, will cause death in five days. This amount of toxin is referred to as the *L₅ dose*. Since the preservation of a standard specimen of toxin was impracticable, Ehrlich employed as a standard in testing, serum of known antitoxic power which was dried and preserved in a vacuum in a cool place, and in the absence of light. With such a standard test-serum any newly prepared serum can readily be compared. An antitoxic serum of which 0.005 c.c. was equivalent to one immunity unit would possess 200 immunity units per c.c. Sera have been obtained of which 1 c.c. has the value of 2,000 units or even more. Within wide limits the principle of constant proportions has been found to hold, thus if the amount of antitoxin is ascertained which neutralises 100 minimum lethal doses of a given toxin,

then ten times as much of the same antitoxin will be required in order to neutralise 1,000 lethal doses of this toxin.

Standardising antitoxin. In order to determine the antitoxic power of a new specimen of antiserum, the following procedure is carried out. In the first place, it is necessary to ascertain the L_+ dose of the given toxin, *i.e.* the smallest amount which, when mixed with the immunity unit of the standard antiserum and injected into a guinea-pig, as above described, will cause death in five days. Then this L_+ dose of toxin is mixed with varying amounts of the new specimen of antitoxin, and the mixtures are injected into a series of guinea-pigs; the largest amount of the serum present in those mixtures which lead to death in five days is equivalent to one immunity unit.

Intracutaneous method Economy in animals and rapidity in obtaining results are effected by this method (Rómer), since one guinea-pig may be injected with a number of mixtures and the results are obtained in two days. The amount of toxin employed in this case is $L_+/500$, the least amount which when mixed with $1/500$ immunity unit of antitoxin and injected in an amount of 0.2 c.c. intracutaneously causes a minimal skin reaction, this is called the $L_+/500$ dose (Glenny and Allan). Other test doses, *e.g.* L_+ , $L_+/100$, are estimated similarly.

Flocculation An interesting physical change resulting from the union of toxin and antitoxin is the flocculation which was studied by Ramon in the case of diphtheria toxin. He found that when graded doses of antitoxic serum are added to a unit volume of a powerful toxin (both in the undiluted state), flocculation occurs in some of the tubes. The tube in which this first appears is commonly the one in which neutralisation is complete, as tested by injection into a guinea-pig. The amount of toxin in c.c. present in this mixture relative to one immunity unit of antitoxin (as defined above) is called the L_f dose of toxin (Glenny *et al.*) Flocculation occurs at room temperature, more rapidly at higher temperatures; but in estimating its first appearance it is preferable to work at the temperature of the laboratory. The phenomenon has come to be of service in standardising diphtheria antitoxin, as it supplies a method much more rapid and more easily carried out than any previously in use. But it is necessary to use the animal test in standardising sera for therapeutic purposes, since there may be discrepancies between the latter and the flocculation reaction, for instance the L_f mixture may sometimes contain excess of toxin when tested by injection into the guinea-pig. The rate at which flocculation occurs (K_f) varies with different specimens of antitoxin, and it has been suggested by Madsen and Schmidt that sera which flocculate toxin rapidly have a greater 'avidity', *i.e.* firmness of combination, than those which act more slowly, and that they will therefore be likely to have a higher curative power. Further investigation of this very important point by Glenny and Barr has indicated that different antitoxin preparations may vary markedly in avidity. This is shown by comparing the action of concentrated and dilute mixtures of the same toxin and antitoxin when a fixed volume (0.2 c.c.) is injected intracutaneously into a guinea-pig. In the case of an antitoxin with weak avidity much more is required to neutralise one L_+ dose of toxin when the mixture is dilute (200 c.c.) than when it is concentrated (2 c.c.), whereas when the avidity is high there is no such discrepancy. The avidity as measured in this way, however, does not necessarily run parallel with the rate of flocculation in the Ramon test. It should be noted further, that neither the L_f nor the K_f value necessarily gives a true indication of the antigenic power *in vivo* of a specimen of toxoid.

Sera of animals immunised with vegetable and animal poisons. It was found by Ehrlich in the case of the vegetable toxins, ricin, and abrin, and also by Calmette and

Fraser in the case of the snake poisons, that the serum of animals immunised against these respective substances had a protective effect when injected along with them into other animals. Ehrlich found, for example, that the serum of a mouse which had been highly immunised against ricin by feeding as described above, could protect another mouse against forty times the fatal dose of that substance. He considered that in the case of the two poisons, antagonistic substances—'antiricin' and 'antiabrin'—were

A corresponding antagonistic
in the blood of animals in the

vegetable and animal poisons,
both as regards their local action and the general toxic phenomena produced by them,
present, as we have seen, an analogy to various toxins of bacteria.

Nature of Antitoxic Action. This subject is only part of the general question concerning the relation of antibodies to their corresponding antigens, but it is with regard to antitoxic action that most of the work has been done. There is now no doubt that the antagonism between toxin and anti-

part of it.

Direct combination. The evidence for this is of several kinds. In cases where the toxin has some definite physical effect, demonstrable *in vitro*, e.g. agglutination, coagulation, or the prevention of coagulation, its action can be annulled by the antitoxin; in such circumstances manifestly no physiological action of antitoxin through the medium of the cells of the body can come into play. The flocculation resulting from the interaction of toxin with antitoxin and the demonstration that the floccules contain both constituents is another clear indication of combination. When toxin and anti-

of the toxin is complete, neutralisation takes place more rapidly in concentrated solutions than in weak; and it is hastened by warmth and retarded by cold. Martin and Cherry showed that diphtheria toxin molecules will pass through a colloid membrane (p. 66), whilst those of the corresponding antitoxin will not. Now if a mixture of equivalent parts of toxin and antitoxin is prepared and is allowed to stand before filtration no toxin is found in the filtrate. Morgenroth has shown that in the case of diphtheria toxin and antitoxin the time necessary for the union to be complete is considerable—about twenty-four hours. Up to this time, mixtures of toxin and antitoxin when injected intravenously, show decreasing degrees of toxicity according to the time they have been kept. On the other hand, when the subcutaneous method of injection is used the time interval has no effect, and this he considered to be due to a catalytic action of the tissues which accelerates the union of the two substances. A striking phenomenon, which points to the temporary reversibility of the combination, was noted by Behring in the case of diphtheria toxin (later confirmed by Glenny and Barr) and studied by Madsen and by Otto and Sachs in the case of botulism toxin. Namely, that when a certain amount of a mixture of toxin and antitoxin was found to be neutral on injection, a fraction of this amount might produce toxic phenomena or even death due to dissociation of the toxin in the greater being the case. Otto and Sachs found that when stand for twenty-four hours, so that combination was complete, the phenomenon no longer occurred. Another experiment also proves the occurrence of direct action between toxin and antitoxin; a neutral mixture of

thermostable snake venom along with thermolabile antivenin becomes toxic if heated soon after mixing, but when the mixture is allowed to stand for a time and is then heated the toxicity does not return. Accordingly, there is little doubt that there are varying degrees of firmness in the union of an antigen and its antibody, and varying periods are necessary for the combination to become complete. It is clear, however, that in general complete combination is not necessarily associated with any destruction of either toxin or antitoxin; actually, the toxin is preserved from deterioration by such combination. Morgenroth found with old mixtures of diphtheria toxin and antitoxin that the combination could be broken down by dilute hydrochloric acid and the two constituents recovered—alkali acts similarly, the union is thus reversible. Ramon *et al* succeeded in dissociating anatoxin by heating an aqueous solution of diphtheria anatoxin-antitoxin floccules at 82°C ., which destroys only the antitoxin. Similarly, by heating toxin-antitoxin floccules at 58° to 60°C . he destroyed the toxin, so that the antitoxin was recovered.

Nature of the combination Although there is agreement as to the direct combination of toxin and antitoxin, there is still much uncertainty as to the exact nature of this union. There may be said to be three chief views—(a)

phenomena correspond to the behaviour of two substances in weak chemical union; and (c) that of Bordet, who regards the combination as of physical nature, corresponding to a process of adsorption between colloids, and not occurring in any definite proportions.

To begin with, it is necessary to state the principal data concerning the quantitative relations according to which toxin and antitoxin react, since the controversy is based on their interpretation; these are the partial saturation effect, the Ehrlich phenomenon and the Danysz effect.

Effect of partial saturation of toxin by antitoxin When to a given multiple (e.g. 100) of the M.L.D. of toxin there is added a varying fraction of the neutralising dose of antitoxin, say one, two, three, etc., tenths, and the toxicity of each mixture is tested on guinea-pigs, it is found in general that larger amounts of antitoxin cause relatively less reduction in toxicity than smaller amounts.

The Ehrlich phenomenon Using one immunity unit of diphtheria antitoxin, Ehrlich determined with any specimen of crude toxin the largest amount of that toxin which could be neutralised completely, so that no symptoms resulted from an injection of the mixture. This amount he called the *limes null* dose, expressed as L_0 . He then investigated the effect of adding to the immunity unit larger amounts of toxin and ascertained the

to be the case, the difference varying according to the particular specimen of toxin examined, but being always considerably more, and often many times the M.L.D.

Danysz effect Danysz established the fact that the amount of toxin neutralisable by a given amount of antitoxin is different according as the toxin is added in several moieties or all at once—in the latter case the amount of toxin neutralisable is greater.

It should be noted that there are two questions which may be independent of each other, namely, (1) does the toxin in any particular case represent a single substance or several; (2) what is the nature of the combination of any one constituent substance and its antibody—is it reversible or is it not?

Ehrlich explained his phenomenon as the result of the presence of toxoids (*vide* p. 69), i.e. toxin molecules in which the toxophore group has become more or less degenerated. He distinguished three possible varieties of such bodies according to the affinity of the haptophore group, namely, prototoxoid with more powerful affinity than the toxin molecule, syntoxoid with equal affinity and epitoxoid with less powerful affinity. The presence of epitoxoids would manifestly explain the phenomenon. The L_{50} dose would represent toxin *plus* epitoxoid molecules each united to antitoxin molecules, and the presence of another M.L.D. of toxin would not result in there being a free fatal dose, but in the excess of toxin taking the place of epitoxoid. Several lethal doses would need to be added before the mixture was sufficient to produce a fatal result—that is, $L_{50} + L_{50}$ equals several M.L.D.'s.

The main contention of Madsen and Arrhenius is that the toxin-antitoxin combination is not a firm one but reversible, and conforms to the law of mass action. For example, in the case of a mixture of ammonia and boric acid (i.e. of a weak base and a weak acid) in solution, there is a constant relation between the amounts of each of the substances in the free condition and the amounts in combination—the combination is reversible, so that if some of the free ammonia were removed a certain amount of the combined ammonia would become dissociated to take its place; further, if to the mixture, in a state of equilibrium, more ammonia or more boric acid were added, part would remain free while part would combine. Accordingly, if toxin and antitoxin behaved in a similar manner, an explanation of the Ehrlich phenomenon would be

very much as a dye becomes attached to blotting-paper. He considers also that there is no definite quantitative relationship in the combination of the molecules of the two substances, different amounts of antitoxin being spread over, as it were, and affecting in varying degree, all the molecules of a given amount of toxin. Recent work of Healey and Pinfield, however, has not fully confirmed Bordet's view. By use of the flocculation method they found that the amount of diphtheria toxin which combines with one unit of antitoxin (i.e. the proportion in the mixture which shows the most rapid flocculation)

called the nitrogen content of the floccules and estimated the proportions due to antitoxin and toxin respectively, they also examined the supernatant fluids for toxic or antitoxic action. It was concluded that in neutral mixtures a fixed amount of antitoxin can unite with different amounts of toxin, the ratio of the latter varying from 1 to 3. Thus there would appear to be a zone of equivalence or neutrality rather than a neutral point.

Full evidence is not yet available on which to decide all the points. The medium in which toxin and antitoxin react is a colloidal one and in all probability both substances are colloids, hence it would be expected that their reactions should exhibit such features as adsorption and flocculation, which are characteristic of reactions between colloids, as emphasised by Bordet. But there is no clear proof of complete absence of fixed combining proportions in the compounds which they form. The existence of toxoids, postulated by Ehrlich, has been demonstrated, and it has been shown that they are produced either spontaneously or under the influence of certain chemical reagents. Glenny *et al.* have found that antitoxin has a greater affinity for toxin than for toxoid and it is known that it may dissociate from either. However, the view that toxoids merely represent toxins which have been deprived of their toxic properties while retaining their combining activity, can hardly be reconciled with the observation of Pons that, unlike tetanus toxin, the corresponding toxoid (anatoxin) lacks combining affinity for brain tissue *in vitro*. The theory of Madsen and Arrhenius regarding the

mass action behaviour of toxin and antitoxin fails to account for the Danysz effect exhibited by mixtures which have been allowed to stand for a considerable time ; here it seems that toxoids must play a part, otherwise the final state of equilibrium would be the same irrespective of the manner in which the mixtures were prepared. The specificity of the reaction between antitoxin and toxin is their most distinguishing feature ; and although many adsorption reactions show definite specificity, this does not throw light on the biological process whereby toxin stimulates the development of antitoxin.

In summary it may be said that direct combination of toxin with antitoxin does occur ; sometimes, probably often, the ' toxin ' contains different toxic derivatives (toxoids) with varying affinity ; and in certain instances the combination has been proved to be reversible, but to what extent this occurs generally remains to be determined. In all cases the outstanding feature is the specific nature of the combination, and of this no satisfactory explanation can as yet be given.

(2) ANTIBACTERIAL ACTION

The procedures in the preparation of antibacterial sera correspond to those in the case of antitoxic sera, but living or, in the early stages, dead cultures are used instead of extracellular toxin. In order to obtain a serum of high antibacterial power it may ultimately be necessary to use a very virulent culture in large doses, for this purpose a virulent culture is obtained from a case of the particular infection, and it is used for inoculation soon after isolation. Its virulence may be further increased by *passage*. This method of obtaining a high degree of antibacterial immunity is specially applicable in the case of those organisms which invade the tissues and then multiply to a great extent, while the toxic effects which they produce are proportionately small in relation to the number of organisms present.

The important result is that the development by such means of a high degree of immunity is accompanied by the appearance in the blood of *protective* substances, which can be transferred to another animal. The law enunciated by Behring regarding immunity against toxins thus holds good in the case of living organisms, as was first shown by Pfeiffer. The latter found, for example, that in the case of the cholera organism, so high a degree of immunity could be produced in the guinea-pig, that 0.002 c.c. of its serum would protect a normal guinea-pig against ten times the lethal dose of the organisms, when injected along with them. Here again is an example of the remarkable potency of the antibodies in an immune serum, which in this case lead to the destruction of the corresponding organisms. The phenomenon of bactericidal action can also be demonstrated *in vitro*. The presence of antibodies in the blood in such actively immunised animals is, of course, a matter of fundamental importance. But it must not be inferred that all the phenomena can be explained in this way. For instance, an antiserum to the anthrax bacillus can be developed and by means of it passive immunity can be transferred to another animal, but the preventive property of the serum is not for bactericidal action demonstrable *in vitro*. Similarly,

to note that every antigen contained in a given species of pathogenic bacteria does not necessarily produce antibodies which are effective in conducting to immunity against the harmful effects which follow infection of the host. Accordingly, the quantitative estimation by *in vitro* methods of antibodies in the blood of an immunised individual, while affording evidence that a

reaction to certain of the bacterial antigens has occurred, does not necessarily afford a measure of the immunity conferred.

Anti-pneumococcal, anti-meningococcal, anti-streptococcal, anti-dysentery, anti-plague, anti-typhoid, anti-cholera sera, etc., have been prepared. In the case of certain organisms there are several distinct antigenic types, e.g. the pneumococcus, meningococcus, streptococcus, dysentery bacillus, etc., and an antiserum to one type has little action, if any, on the other types of the same species.

Properties of Antibacterial Sera. We have here to consider the three main actions mentioned above, namely, (a) bactericidal and lysogenic or bacteriolytic action, (b) opsonic action, and (c) agglutination and the closely allied precipitation. Of these the two first are those chiefly concerned with the protective property of an antibacterial serum. These various properties are due to the presence of corresponding antibodies in the serum—immune-bodies, opsonins, agglutinins, etc.—but, as already stated, it is not to be assumed that these are separate and distinct substances; they are in fact recognisable only by their effects. There is also (d) antiaggressin action.

(a) Bactericidal and Bacteriolytic Action. Pfeiffer found in the case of certain organisms, e.g. virulent cholera vibrios, that if they were injected into the peritoneal cavity of a guinea-pig highly immunised against them, they lost their motility almost immediately, gradually became granular, swollen, and then disappeared in the fluid—these changes constitute what is now generally known as 'Pfeiffer's phenomenon' or bacteriolysis. The phenomenon occurs also *in vitro* with immune serum in the fresh state, but not after heating at 55° C. It was subsequently shown by Metchnikoff and by Bordet that bacteriolysis occurred outside the body on the addition of fresh peritoneal fluid or normal serum to heat-inactivated (55° C.) immune serum. It was also found that an antiserum heated even up to 70° C. for an hour produced the reaction when injected together with the corresponding organisms into the peritoneum of a normal animal. The outcome of these and subsequent researches was to show that when an animal is immunised against an organism, there appears in its serum an antibody, which is generally known as *immune-body*, *amboceptor* (Ehrlich), or *substance sensibilisatrice* (Bordet), it is comparatively stable, resisting usually a temperature of over 55° C. for an hour. It cannot produce the destructive effect alone, but requires the addition of an unstable, thermolabile substance normally present in the plasma and serum, which bears various names—*complement* (Ehrlich), *alexin* or *cytase* (French writers). Complement is not increased in amount during the process of immunisation. The complement in acting on bacteria *plus* immune-body is fixed, or used up, in definite quantities.

The phenomenon of serum-bacteriolysis is well marked only in the case of certain organisms when an animal is highly immunised against them; the typhoid and cholera group are outstanding examples. In other cases the bactericidal effect of serum may occur without lysis of the bacteria, though other structural changes may be produced. In still other instances, e.g. the antisera to staphylococci, streptococci, plague bacilli, etc., a bactericidal effect may be wanting, nevertheless it may be shown that an immune-body is developed in the process of immunisation. This may be done by observing the increased amount of complement which is fixed through the medium of the antiserum (immune-body), sensitised red corpuscles being used as the test for the presence of free complement. The method is described on p. 94.

The all-important action of the immune-body is thus to bring an increased amount of complement into union with bacteria; whether death of the

bacteria will result or not will depend ultimately on their sensitiveness to the action of the particular complement.

It is to be noted that in the case of a bactericidal serum there is an optimum amount of immune-body which gives the greatest bactericidal effect with a given amount of complement. If this amount of immune-body be exceeded, the bactericidal action becomes diminished and may be practically annulled. This result, which is generally known as the 'Neisser-Wechsberg phenomenon', has been the subject of much controversy, and cannot yet be said to be satisfactorily explained, it is apparently of the nature of a 'zone phenomenon' (cf p. 110) (Regarding some theoretical considerations as to the therapeutic applications of antibacterial sera, *vide* p. 119.)

In certain instances a normal serum may possess bacteriolytic or bactericidal action on various bacteria, *e.g.* *V. cholerae*, *B. typhosus*, *B. dysenteriae*, etc., and this may be shown to be due to natural immune-body acting along with complement. In other words, what is observed in the state of active immunity represents a further development of a condition normally present (*vide* Natural Immunity)

Estimation of the bactericidal action of serum. This may be carried out by various methods, of which the following are representative examples

(a) *Method of Neisser and Wechsberg* A series of small plugged sterile tubes is taken, and to each is added 0.5 c.c. of neutral diluent (0.85 per cent NaCl solution was used

the contents of each agar tube are mixed and poured out into a sterile Petri plate. The Petri plates are placed in the incubator for a suitable period (forty-eight hours). The (Gelatin can be substituted for the agar in the amount of serum is ascertained which is

hours' broth culture is used, and various

prepared and treated in the same way. The tubes are then placed in the incubator for eighteen to twenty-four hours at 37° C., and at the end of that time the contents of each are tested for sterility by means of cultures. In this way the greatest dilution in which

The number of bacteria per c.mm. (div), and thus the total number of be readily calculated

can be carried out rapidly and gives of six or more tubes containing 0.1 or of varying density—it is convenient to use decimal dilutions, *e.g.* 1/100, 1/1,000, etc., of a standard suspension

of a twenty-four-hours' culture. In making these dilutions the pipette should be changed

tubes at 37° C. for four hours or longer another plate is inoculated in the same way from each tube. After time has been permitted for growth to occur on the plates at 37° C. the

results are compared. Bactericidal action is shown by the occurrence on the second plate of only scanty colonies or no growth in a series of concentrations higher than those yielding the same result on the original plate. The approximate degree of bactericidal action is determined by the difference between the end points of growth in the series of stroke cultures before and after incubation.

The bactericidal action of whole blood can be estimated by this method, defibrinated or preferably heparinised blood being tested in place of serum (see Miles and Misra). The mixtures must be shaken repeatedly during incubation to prevent sedimentation of the blood cells. If the incubation is prolonged some form of shaking machine is required which will agitate the tubes just sufficiently to maintain the blood in suspension (Todd). Other methods have been used by Wright, and Robertson and Sia.

(d) *Method for an immune serum* To analyse the bactericidal properties of the serum of an animal immunised against a particular organism, it should in the first place be heated at 55°C in order to destroy the normal complement. Then to each of a series of sterile tubes containing diluent, so as to maintain a constant final volume, we add (a) a quantity of normal unheated serum insufficient of itself to destroy the bacteria, to act as complement, (b) a given amount of the bacterial culture, and (c) varying amounts of the heated immune serum—0.1, 0.01, 0.001, etc., c.c. The mixtures are then incubated and a fixed amount of each is plated, as described for Neisser and Wechsberg's method. In this way is found the quantity of the immune serum which gives the maximum bactericidal action.

HÆMOLYTIC SERA The phenomena of lysogenesis are, however, not peculiar to the case of solution of bacteria by the fluids of the body, but hold also for other cellular elements (red corpuscles, leucocytes, etc.) when these are introduced into the tissues of an animal as in the process of immunisation. Of the cytolytic or cytotoxic sera so produced the hæmolytic have been most fully studied, and, owing to the delicacy of the reaction and the ease with which it can be observed, have been the means of throwing much light on the process of lysogenesis, and thus on one part of the subject of immunity. A short account of their properties may now be given.

It has long been known that in some instances the blood serum of one animal has, in a certain degree, the power of dissolving the red corpuscles of another animal of different species; in other instances, however, this property cannot be detected. Bordet showed that if an animal received repeated injections of the red corpuscles of a different species, the serum of the former acquired a marked hæmolytic property towards the corpuscles of the latter, this being demonstrated when the serum was added to the corpuscles and the mixture kept for a time at 37°C . He found that the hæmolytic property disappeared when the hæmolytic serum was heated at 55°C .; but (as with a bacteriolytic serum) lytic action was regained on the subsequent addition of some fresh serum from a non-treated animal. Ehrlich and Morgenroth analysed the phenomena in question, and showed that the specially developed and heat-resisting substance, 'immune-body', entered into combination with the red corpuscles at a comparatively low temperature, namely, at 0°C .; whereas complement did not combine at this temperature. In this way a method is supplied by which the immune-body can be removed from a hæmolytic serum while the complement is left. They supposed that immune-body combined with the complement, though the combination is not firm and only occurs at higher temperatures—best about 37°C . They therefore regarded the immune-body as a sort of connecting-link between the red corpuscle and the complement, hence the term 'amboceptor' afterwards applied to it by Ehrlich. It may be stated, however, that the direct union of complement and immune-body has not been conclusively demonstrated. Muir and Browning, for example, found that when fresh serum is passed through a Berkefeld filter, complement is largely retained by the filter, whereas immune-body passes through practically unchanged; and that if a mixture of complement and immune-body be made and filtered at a

temperature of 37°C , the amount of immune-body which passes through is not diminished, whereas it would be if it had united with the retained complement. Accordingly this method did not afford evidence of the direct union of immune-body and complement. Bordet holds that the immune-body acts merely as a sensitising agent—hence the term *substance sensibilisatrice*—and allows the complement to unite. It is quite evident from his writings, however, that he does not mean, as is often assumed, that the immune-body causes some lesion in the corpuscle which allows the complement to act, but simply that it produces in the molecules (receptors) of the red corpuscles an avidity for complement. All that can be said definitely at present is that the combination antigen *plus* immune-body takes up complement in firm union, while neither does so alone. Complement acts quantitatively, thus the smallest amount can be ascertained which will just suffice to cause lysis of a unit volume of suspension of red blood cells sensitised with immune-body; this is known as the 'minimum hæmolytic dose' (M.H.D.) of the complement-containing serum. Even after the corpuscles are laked with water the receptors are not destroyed, and the corpuscles which have been laked can still take up immune-body as readily as the intact corpuscles do. In fact the red corpuscles can take up much more immune-body than is necessary for their lysis, and Muir found in one case studied, that each further dose of immune-body led to the fixation of more complement, so that as many as ten times the hæmolytic dose of complement might thus be used up. It is a matter of considerable importance that the union of immune-body and red corpuscles can be shown to be reversible. As was found by Morgenroth and Muir independently, if corpuscles treated with several doses of immune-body and then repeatedly washed in salt solution, be mixed with some untreated corpuscles and allowed to remain for an hour, sufficient immune-body will pass from the former to the latter, so that all become lysed on the addition of sufficient complement. Muir showed also that the union of immune-body with red corpuscles was not increased in firmness after twenty-four hours. The combination of complement, on the other hand, is usually of very firm nature.

Hæmolytic sera are of great service in the study of the question of specific-

usual absorption tests, for example, the antiserum to ox's red cells combines with sheep's corpuscles. A close analogy holds to what has been established in the case of agglutinins. It is further of great interest to note that by the injection of foreign red corpuscles into an animal its serum not only becomes hæmolytic, but also in many cases after heating at 55°C possesses agglutinating and opsonic properties towards the red corpuscles used. These facts show how close an analogy obtains between antibacterial and hæmolytic sera, and how important a bearing hæmolytic studies have on the questions of immunity in general.

In addition to species specificity, individual specificity can also be demonstrated sometimes, since the injection of red cells derived from other individuals of the same species will develop 'isolymins', although an 'autolysin' active against the individual's own corpuscles is not produced.

Methods of hæmolytic tests. A hæmolytic serum is usually prepared by injecting, e.g. intraperitoneally, the red corpuscles of an animal into one of different species, the corpuscles of the ox or sheep are most frequently used, and the rabbit is the most suitable animal for injection. The corpuscles ought to be completely freed from serum by washing

at 55° C. on three successive days; it is, of course, devoid of complement. The test amount of corpuscles is usually 0.5 c.c. of a 5 per cent. suspension of washed blood (see above) in 0.85 per cent sodium chloride solution, that is, the corpuscles of 5 c.c. blood completely freed of serum, and then salt solution added to make up 100 c.c. (or 3 c.c. of packed blood sediment plus 97 c.c. saline). In any investigation it is necessary to ascertain the minimum hæmolytic dose (M.H.D.) of the immune-body and of the complement to be used. As complement does not increase during immunisation, the hæmolytic dose of the fresh immune serum will come far short of representing the amount of immune-body present.

In testing the dose of immune-body, the fresh serum to be used as complement must be devoid of hæmolytic action (in the present instance rabbit's serum will be found suitable), and more than sufficient to produce lysis with immune-body is added to each of a series of tubes. Instead of rabbit's serum it is preferable to use guinea-pig's serum as the source of complement, since it is much more active. The latter often contains some natural

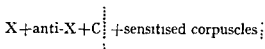
covered by centrifuging and is pipetted off, it is now devoid of any lytic action by itself, but contains the complement, 0.05 or 0.1 c.c. of this treated serum is added to each tube of the test amount of corpuscles in estimating the dose of immune-body. Varying amounts of immune-body (which should be diluted with saline in order to permit of accurate measurement) are added to the tubes, the contents are shaken, and incubated at 37° C. for one and a half hours. The amount of lysis is then noted. The smallest amount of immune-body which gives complete lysis is, of course, the M.H.D.; this may be as low as 0.0005 c.c. for the test amount of corpuscles. An immune-body for sheep's red corpuscles can be developed similarly by injections of washed sheep's blood. Since guinea-pig's serum seldom has much natural hæmolytic action on these corpuscles it can be used without previous treatment with the blood at 0° C.

To estimate the M.H.D. of complement, proceed in a corresponding manner; to each of a series of tubes containing the test amount of corpuscles add several (at least five) doses of immune-body, and then to the several tubes different amounts of complement. Nearly maximal lysis is obtained after one hour's incubation. The amount of complement necessary for lysis varies somewhat according to the amount of immune-body used, being smaller with several doses of the latter than with a single dose; in estimations of the dose of complement, it is accordingly advisable to use the optimum amount of immune-body (about five hæmolytic doses in the present instance). The activity of a serum as complement varies considerably, and each sample must be tested separately. It can be preserved for a considerable time at or near its original strength if it be kept frozen. Even if this be done, however, the strength of the complementing serum must be titrated at the commencement of every experiment in which it is employed. Commonly the M.H.D. of fresh guinea-pig's complement for 0.5 c.c. ox or sheep corpuscle suspension is 0.005 or 0.0075 c.c. Corpuscles treated with sufficient immune-body to produce complete lysis on the addition of complement are usually spoken of as *sensitised corpuscles*.

COMPLEMENT-FIXATION REACTION OF BORDET AND GENGOU

From the facts given above it follows that sensitised red corpuscles, *i.e.* corpuscles treated with immune-body, may be made to serve as an indicator for complement. If an antibacterial immune-body is present in a serum heated at 55° C., this serum along with the corresponding bacteria will lead to the fixation of complement, and thus prevent hæmolysis when sensitised corpuscles are added later. If we represent the bacteria, or rather the receptors in the bacteria, by X, the immune-body by anti-X, and the

complement by C (normal serum, say, of a guinea-pig) we may represent the method of experiment by the following scheme.



(The vertical dotted line represents a period of incubation for one and a half hours at 37° C.)

If lysis of the sensitised corpuscles does not occur after incubation at 37° C., then the complement has been fixed and an immune-body has been shown to be present, provided that a suitable control shows that the bacteria alone, without immune-body, do not fix sufficient complement to interfere with lysis. The reaction may also be controlled by substituting for the immune serum heated normal serum of the same species; absence of complement fixation under these conditions shows that there is no natural antibody present.

This method has now been extensively used for demonstrating the presence of immune-bodies in the blood of patients infected with a particular organism. It has also been applied to determine whether a suspected organism is really the cause of a disease, for if the organism in question gives with the serum of the patient fixation of complement, then there is a strong presumption that it is the infective agent. The antigen consists of broth cultures or suspensions in saline of cultures of the organisms on solid medium, which have usually been killed by heating. The optimum amount of organisms to be employed must be ascertained in every instance, but the test amount of bacterial suspension should not by itself inhibit more than one or two doses of complement. It is essential that antisera which are to be tested in this way should not have been obtained from animals injected with cultures grown on foreign blood or serum, since antibodies generated by the latter may lead to fallacies; but other constituents of culture media may also be antigenic (*vide* p. 78). Complement fixation may be said to occur when the mixture of antigen and serum inhibits more complement than the sum of the amounts inhibited by each of these reagents separately, but the phenomenon is clearly present when the mixture of antigen with the serum of an individual infected or inoculated with the organism in question inhibits complement to a greater degree than does a similar mixture containing serum from an uninfected individual. Different specimens of complement of equal hæmolytic power may differ in their *derivability*, *i.e.* capacity for being fixed by a given mixture of antigen *plus* antibody.

In carrying out complement-fixation tests care must also be taken that the source of complement, usually guinea-pig's serum, does not contain a natural antibody for the antigen employed. In order to make sure of this a control is included in the series, in which the effect on complement of the antigen by itself is determined. It has been found in the case of *B. typhosus* and other organisms that under such conditions a small amount of complement may fail to be fixed, whereas fixation occurs in the tubes containing larger amounts (Dunlop). This disturbing property of the guinea-pig's serum may usually be removed without damaging the complement by keeping it at 37° C. for several hours or by treatment with charcoal.

Fixation of complement occurs frequently when an antibody is added in suitable proportion to the corresponding antigen (bacteria, viruses, tissue cells, protein solutions such as serum, etc.) or hapten (the Wassermann reaction is an analogous phenomenon). But all antigen-antibody mixtures do not have the capacity to fix complement. In the case of antigens or haptens

which are soluble, complement fixation and precipitation are frequently associated, but the two phenomena do not necessarily proceed in parallel. According to Goldsworthy there is an optimal size of the particles for complement fixation. Another determining factor may be the species of animals from which the antibody and the complement are derived.

Nature and mode of action of complement. Complement is relatively unstable, being destroyed in 30 to 60 minutes at a temperature of 55° C.; also it deteriorates in a few days at room-temperature. It has been a disputed point whether there are several distinct complements in a normal serum, with different relations to different immune-bodies, for which Ehrlich and his co-workers have brought forward a certain amount of evidence, or whether, as Bordet holds, there is a single complement, which may, however, show slight variations in behaviour towards different immune-bodies. There is no doubt, however, that all the complement molecules in a serum are not the same. For hæmolysis the immune-body and the complement need not be derived from the same species. As a rule sensitised red cells are not readily lysed by the complement of their own species. In general, guinea-pig's serum is the most active complement for lysing blood corpuscles of other mammals. It has been held by workers of the French school that complement does not exist in the free condition in the blood, but is liberated from the leucocytes when the blood is shed; but there are many facts against such a view. For instance, it was shown by Muir and McNee that the introduction of immune-body into the circulation of the corresponding animal leads to a lysis of red corpuscles by means of complement, that the lysis may go on gradually for a considerable period of time and, further, that immune-body may become dissociated from red corpuscles and combine with others, the latter then undergoing lysis. These facts and many others speak strongly in favour of the view that complement exists in the free condition in the circulating blood; but its source is unknown. There is no evidence that the leucocytes contain complement.

Observations by Ferrata, Brand, and others have shown that complement is not a single substance, but is really made up of several components. Thus dialysis or treatment with carbon dioxide or weak hydrochloric acid leads to precipitation of part of the serum globulins. The component in the precipitate, when the latter is separated and dissolved in saline, unites directly with sensitised corpuscles, and then that in the separated fluid enters into combination. Hence they have been called 'middle-piece' and 'end-piece' respectively. Neither component by itself produces the complementing effect, but together they may act like the original serum. The separation, however, is not always successful. Other components of complement have also been described, serum which has been rendered inactive, e.g. with a suspension of yeast cells, may have its complementing activity restored by adding a small amount of serum heated at 55° C. ('third component'), which possesses neither middle-piece nor end-piece properties. Again, Gordon, Whitehead and Wormald have shown that a 'fourth component' of complement is destroyed by treating the serum with a minute amount of ammonia, and that reactivation occurs on adding to it a specimen of serum from which the third component has been removed. The work of Browning and Mackie, who fractioned complement-containing serum with ammonium sulphate as well as by treatment with carbon dioxide, demonstrated that the constitution of complement is even more complex than the above results would indicate. Complement, as tested by bactericidal action, is found also to have a complex constitution. Complement action seems to depend on the presence in suitable amounts of certain cations, especially magnesium (Mayer *et al.*).

Heidelberger *et al* estimated chemically the amount of nitrogen contained in precipitates formed when a serum reacts with the homologous antiserum in the presence or absence of complement. In the former case the nitrogen was increased; and by this means they measured the complement content of a serum gravimetrically. Because of the irregular degree of hæmolysis effected by identical volumes of a highly diluted specimen of complement when added to a fixed amount of sensitised red cells, Lominski has concluded that complement, like bacteriophage, is of particulate nature.

Conglutination. Fresh serum of the ox—and certain other species—when added to red blood corpuscles (or bacteria) sensitised with immune-body brings about intense agglutination. The same result follows the addition of heat-inactivated ox serum along with a trace of complement. It appears that the effect is due to a special property of the ox serum—conglutinin, and by means of this phenomenon as the indicator it is possible to demonstrate the action of antibody which would otherwise escape detection.

Normal Serum Hæmolysins. These can be shown in some cases to be of the same nature as those of an immune serum, that is, complement and the homologue of an immune-body can be distinguished. For example, guinea-pig's serum is hæmolytic to ox's corpuscles; if a portion of serum be heated at 55°C , the complement will be destroyed; if another portion be treated with ox's corpuscles at 0°C , the natural immune-body will be removed and only complement will be left. Neither portion is in itself hæmolytic, but this property becomes manifest again when the two portions are mixed.

Blood Groups. These groups were discovered in human subjects by Landsteiner

... same time those natural antibodies which do not correspond with the individual's own red cells ... the A+B principle is easily ... presence of these antigens and antibodies may lead to serious accidents after blood transfusion if unsuitable blood is used, that is, especially a specimen for which the

injecting the corresponding blood cells into rabbits. Another class of antigens, termed Rh (present also in the red cells of *Rhesus* monkeys), are found in the corpuscles of a proportion of human subjects. Their practical importance consists in the fact that if a woman who lacks them produces a foetus which has inherited the Rh factor from its father, the antigen may traverse the placenta and stimulate an immunity reaction in the

Forssman's Hæmolytic Antibody. It has been shown by Forssman that a lysis for sheep's red corpuscles can be developed in the rabbit by the injection of emulsions of tissues of certain animals, *e.g.* guinea-pig, horse, etc. The antigens in these tissues are known as *heterogenetic* and the corresponding antibodies as *heterophile*. The nature of such antigens is still unknown, though there is evidence that they are combinations of carbohydrates, perhaps with lipoids. The antibodies along with extracts obtained by treating the organs with alcohol give reactions *in vitro*, *e.g.* fixation of complement and flocculation, that is, the extracts act as haptens. It has been found impossible, however, to obtain heterophile antibodies by the injection of separated lipoids alone, but a mixture of the latter with foreign serum (*e.g.* pig's) develops in rabbits an antibody which gives reactions with the lipoids (Landsteiner and Simms). Various bacteria contain heterogenetic antigen, *e.g.* Shiga's dysentery bacillus.

Cytotoxic Sera. In addition to hæmolytic sera, antisera have been obtained by the injection of leucocytes, spermatozoa, ciliated epithelium, liver cells, nervous tissue, etc. The laws governing the production and properties of these are identical, that is, each serum exhibits a specific property towards the cells used in its production—*i.e.* dissolves leucocytes, immobilises spermatozoa, etc. The specificity is, however, not so marked as in the case of sera produced against red blood corpuscles; thus a serum produced against tissue cells is often hæmolytic, this is probably due to various cells of the body having the same receptors. Here again, when the antiserum produces no destructive effect on the corresponding cells, the presence of an immune-body may be demonstrated by the increased amount of complement which is taken up through its medium. It may also be mentioned that each antiserum usually exhibits toxic properties towards the animal whose cells have been used in the injections, *e.g.* a hæmolytic serum may produce a fatal result, with signs of extensive blood destruction, hæmoglobinuria, etc., *i.e.* it is hæmotoxic for the particular animal; a serum prepared by injection of liver or kidney cells has been found to produce on injection necrotic changes in the respective organs of the species of animal whose cells were used. These are mentioned as examples of a very large group of specific activities.

(b) **Opsonic Action.** The main facts with regard to phagocytosis in relation to the ties of the cytosis in

occurrence in the susceptible animal, and showed further that the acquisition of immunity against a particular organism was attended by the appearance of active phagocytosis towards it. He explained the phenomena at first by supposing some essential change in the leucocytes, and later, when the favouring action of serum came to be recognised, by supposing that the serum antibodies acted on the leucocytes or, to use his term, acted as 'stimulins'. Variations in the activity of the leucocytes have been observed in certain conditions, but these are not of specific character, that is, are not specially directed towards the organism against which immunity is acquired. It is now recognised also that so far as specific variations are concerned, the leucocytes are an indifferent factor. All the important variations in phagocytosis in relation to immunity may be said to depend on the opsonic properties of the serum on the one hand, and on the virulence of the organism on the other.

The presence of a substance in an immune serum which makes the corresponding organism sensitive to phagocytosis was first demonstrated by Denys and Leclef in 1895, in the case of an anti-streptococcal serum. They also showed that the serum produced this effect by acting on the organism, not on the leucocytes. It is, however, chiefly due to the researches of Wright and his co-workers that attention has been directed to the conditions under which phagocytosis of organisms by the polymorphonuclear leucocytes occurs. Wright and Douglas first showed that the phagocytosis of staphylococci by leucocytes depended on a body in normal serum which became fixed to the cocci and altered them so that they were then ingested by the phagocytes. To this they gave the name of 'opsonin'. There is no phagocytosis of cocci by leucocytes washed in salt solution; normal serum heated to 55° C. is also without effect in inducing this phenomenon. ('Spontaneous' phagocytosis of non-virulent organisms by washed leucocytes in the absence of serum may occur in certain instances, however.) With regard to the mode of action of opsonin, Wright and Douglas could not demonstrate that it had any effect on the leucocytes. On the other hand, if bacteria were exposed to the fresh serum, and they were freed from the excess of serum and then exposed to leucocytes, also washed free from serum, they were readily taken up by the cells. While the investigation of opsonic action has been carried out chiefly *in vitro* with polymorphonuclear leucocytes, it is known that the macrophages of the reticulo-endothelial system behave

similarly. Virulent organisms, such as pneumococci, when injected intravenously, disappear more rapidly and permanently from the circulation of actively or passively immunised animals than of susceptible ones. It has been found by microscopic examination and by making cultures that the organisms accumulate especially in the liver and spleen in the mononuclear phagocytes. Although pathogenic organisms of various species can survive phagocytosis, it has been established that in many cases they are destroyed both *in vivo* and *in vitro* by polymorphonuclear leucocytes and macrophages. It remains uncertain whether the function of opsonins is merely to prepare the organisms for ingestion, their destruction then following by mechanisms of the phagocytes. Opsonins also damage cells by their direct opsonic action is the

Estimation of opsonic action The opsonic property of a specimen of serum may be estimated in the following way (Leishman's method as modified by Wright *et al.*). A uniform suspension in saline of a young culture of staphylococci is prepared. Also a specimen of leucocytes is obtained as follows: some drops of freshly drawn blood are added to a solution containing 1.0 per cent sodium citrate and 0.6 per cent sodium chloride, mixed and centrifuged, the supernatant fluid being removed and the sediment resuspended in fresh saline and again centrifuged, the upper layer, which is rich in leucocytes, finally being pipetted off. Equal volumes of bacterial suspension, leucocytes, and serum are mixed thoroughly and drawn into a capillary pipette, which is placed at 37° C. for fifteen minutes. (It is advantageous to maintain a uniform mixture mechanically by keeping the pipette in continuous motion during incubation.) At the end of this time films of the mixture are made on microscope slides and stained, e.g. with Leishman's fluid. The number of cocci contained in a sufficiently large series of consecutive leucocytes (at least 100) is counted and the average number per leucocyte estimated (the phagocytic index). In order to compare a patient's serum with that of a healthy person both are treated in parallel as described above, human leucocytes being used. The phagocytic index of the former is divided by that of the latter, the result is called the 'opsonic index'. Another method consists in ascertaining the phagocytic indices obtained with a series of dilutions of each serum. These dilutions are selected which yield the same phagocytic index with the patient's and with the normal serum, the reciprocal of the former divided by the reciprocal of the latter is the opsonic index. The opsonic index of a patient's serum in opson serum (1:20, 1:30, 1:40, 1:50, 1:60, 1:70, 1:80, 1:90, 1:100) is determined in Wright's procedure.

A consecutive series of 50 leucocytes is estimated, the extinction point being noted also.

Thermostable opsonins are estimated similarly, except that the serum is heated beforehand, say at 55° C., for a half to one hour.

Differences between normal opsonins and immune opsonins It has been shown that the opsonic action of the serum against an organism is increased by the process of immunisation, and the opsonic index represents the degree of immunity in one of its aspects, as already explained. In an immune serum, however, an opsonin may still be present after the serum is heated at 55° C., as was demonstrated by G. Dean and others, and Muir and Martin showed that this thermostable immune opsonin (bacteriotropin of Neufeld) has all the specific characters of antibodies in general. On the other hand, they found that the thermolabile opsonin of a normal serum has quite different properties. For example, the opsonic effect of a normal serum for a particular organism may be removed by treating the serum with other bacteria, in other words, the thermolabile opsonin of normal serum does not possess the specific character of the opsonin developed in the process of immunisation. They found, further, that various substances or combinations of substances which act as 'complement absorbers' also remove the opsonic property from a normal serum, while they have no effect on an immune opsonin. The opsonic property of fresh normal serum, however, is unaffected by treatment of the serum with certain reagents which abolish complement

action, such as ammonia (which destroys the 'fourth component' of complement, p 96), hydrochloric acid, caustic soda, or congo red (Gordon, Whitehead, Wormall, and Thompson). Accordingly, there is not a complete identity between the action of complement and normal opsonin.

The non-specific effect of thermolabile normal opsonin is clearly shown by the fact that particles of carmine and other substances become opsonised by the action of normal serum. It is to be noted, however, that in certain cases there have been found in normal serum traces of substances which can be activated by thermolabile opsonin after the manner of immune-body and complement (as seen in the hæmolytic action of a normal serum, p 97); to this extent the opsonic effect of a normal serum may have some degree of specificity. From this and other facts some observers have attempted to explain the whole of opsonic action according to the scheme of immune-body *plus* complement as seen in hæmolysis. This, however, is not justifiable, since normal thermolabile opsonin can, as already seen, act by itself, as can also the specific immune opsonin after normal opsonin has been destroyed by heating. The subject is one of considerable complexity, but it may be said that the most important cause of increase in the opsonic effect observed in infections is the specific bacteriotropins, though the presence of immune-body may play a part by leading to the union of more normal opsonin.

Immune opsonins (bacteriotropins). The development of increased opsonic action of the serum may be regarded as the most important factor in active anti-bacterial immunity, since, as we have seen, an enhanced direct bactericidal action occurs only in the case of a limited number of organisms. The increased opsonic action is due to antibodies which act either with or without complement—opsonic immune-bodies or bacteriotropins (immune opsonins) respectively. The latter are of special importance, as they can act in high dilution and are effective in situations where complement has been used up, as is often the case. Moreover, comparative observations show that there is a close correspondence between phagocytosis *in vitro* and *in vivo*. In the immune animal, accordingly, the antibodies lead to ingestion of the bacteria, and this is often, though not invariably, followed by their destruction, enhanced powers of dealing with the bacteria thus result.

Virulence of organisms in relation to opsonic action. An important factor in relation to phagocytosis is the state of the organisms as regards virulence. A relatively non-virulent organism may be susceptible to phagocytosis when acted on by normal serum (or even in the absence of serum altogether), but a more virulent strain may require the action of an immune serum, this is seen in the case of certain streptococci. Similarly, organisms from a culture may be more resistant to opsonic action and more virulent at an early stage of growth than later. The factors on which virulence depends are various and are imperfectly known, but in some organisms, *e.g.* pneumococcus and the anthrax bacillus, increased virulence is associated with the development of capsules. When anthrax bacilli are introduced into the body there may be active phagocytosis for some time, but then a capsulate race may appear which is not susceptible to opsonic action either in the living body or *in vitro*. Capsule formation in some way protects the organism against opsonic action, but increased virulence may occur without it; for example, some strains of virulent streptococci are without capsules, while in others increased virulence is associated with capsule formation. There is also evidence that in some cases virulent bacteria produce aggressins or leucocidins which have an antagonistic action on the phagocytes.

Nature of opsonic action. The union of opsonising substance, whether specific or non-specific, has been shown to result in certain physical changes

in the bacteria. The subsequent phagocytosis of them by the leucocytes occurs with remarkable rapidity, as shown by the fact that several bacteria may be taken up by a leucocyte within a minute. It is now recognised that both the bringing of the bacteria into contact with the leucocytes and the subsequent ingestion of them depend on physical processes. The bacteria are drawn together towards the leucocytes by a process allied to agglutination, and thus are seen to form rings around them (Ledingham). Increased adhesiveness of the bacteria to the leucocytes is produced by opsonic action, and this means diminished surface tension at the point of contact, the bacteria thus becoming merged in the leucocyte protoplasm quite apart from amoeboid activity. Tannic acid has been found to opsonise organisms, and substances in general which possess tanning properties act in this way (Gordon and Thompson). These facts have been established with regard to *in vitro* experiments, but the same principles no doubt hold with regard to phagocytosis in the body. At the same time it must be recognised that leucocytes in the tissues move towards the bacteria under chemotactic attraction, and their emigration from the vessels seems capable of explanation only in this way. Such chemotactic attraction has been demonstrated also *in vitro* in certain instances.

SUMMARY OF THE MECHANISM OF OPSONIC ACTION

(1) Pathogenic organisms *in vitro* are usually not ingested by phagocytes (polymorphonuclear leucocytes) in the absence of serum.

(2) Opsonins, the substances in serum which promote phagocytosis, act by altering the organisms; they do not have any direct effect on the leucocytes.

(3) The opsonins of normal and immune sera may be distinguished.

(4) Normal opsonins are thermolabile at 56° C. They are non-specific, since treatment of unheated serum with one species of organisms removes the opsonins for other species; they are related to, but not identical with, bacteriolytic and hæmolytic complement. Thus many procedures which remove the complementing action of serum also deprive it of normal opsonin. On the other hand, serum which has been deprived of the fourth component of complement, still has opsonic action.

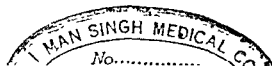
(5) Immune opsonin (bacteriotropin) which is developed in the serum as the result of immunising an animal with the organism in question, has the properties of a specific thermostable antibody.

(6) The opsonic effect of complement *plus* immune opsonin may be greater than the sum of the opsonic actions of each component separately.

(7) In certain cases the opsonic property of a normal serum is due to thermostable constituents.

(c) **Agglutination and Precipitation.** Charrin and Roger in 1889 observed that when *Bacillus pyocyaneus* was grown in the serum of an animal immunised against this organism, the growth formed a deposit at the foot of the vessel, whereas a growth in normal serum produced a uniform turbidity.

Shortly after the discovery of agglutinins, Kraus found in the case of the organisms of typhoid, cholera, and plague, that the antiserum not only caused agglutination, but when added to a filtrate of the corresponding culture produced a cloudiness and afterwards a precipitate. To the substance in the immune serum which brought about this effect he gave the name of



precipitin. Subsequent study has shown that this phenomenon is closely related to agglutination.

Agglutinins. It was known that the serum of convalescents from typhoid fever could protect animals to a certain extent against typhoid infection, and, in view of the facts experimentally established, it appeared a natural proceeding to inquire whether such serum possessed an agglutinative action and the stage of the disease at which it appeared. The result, obtained independently by Grünbaum and Widal, but first published by the latter, was to show that the serum possessed this specific action shortly after infection had taken place; in other words, the development of this variety of antibody can be demonstrated at an early stage of the disease. Agglutinins are developed generally in bacterial infections, though the degree of their production and the facility with which they can be demonstrated vary greatly in different cases. Details will be found in the chapters dealing with individual infections. Furthermore, the phenomenon is not peculiar to bacteria; it is seen, for example, when an animal is injected with the red corpuscles of another species, *hæmagglutinins* appearing in the serum, which have a corresponding specificity, as was first shown by Bordet.

Agglutination has usually been regarded as a phenomenon which in itself does not play any part in conducing to the state of immunity. In the case of rabbits whose blood was richly infected with *pneumococci*, Bull, however, found that the intravenous injection of anti-pneumococcal serum of homologous type brought about immediate clumping of the cocci and their phagocytosis by polymorphonuclear leucocytes in the capillaries of the liver, spleen, and lungs. He concluded that the agglutination is an essential process along with the phagocytosis in bringing about protection. The administration of excessive doses of antiserum, however, caused the formation of clumps of the cocci so large that they escaped phagocytosis and thereupon multiplied, causing a fatal infection. The agglutinating action of antibody appears also to account for the fact observed by Rich that *pneumococci* injected into the skin of an immunised rabbit remain localised, whereas in a susceptible animal they rapidly spread through the tissues.

Varieties of agglutinins—flagellar, somatic, Vi. Smith and Reagh, Weil and Felix, and others have shown that in the case of many motile bacteria agglutinins are developed against both the flagella and the bodies of the bacteria—*flagellar* or *H*, and *somatic* or *O* agglutinins respectively, and these two types have a different significance. The flagellar agglutinins lead to a rapid formation of loose flocculent clumps, while the somatic agglutinins act more slowly and the clumps are smaller and denser. The somatic antigens or agglutinogens are demonstrated in the case of motile bacilli by means of non-motile variants or by the use of suspensions which have either been heated at 100° C or treated with alcohol—these procedures having the effect of killing the bacteria. The flagellar antigen is demonstrated by the use of a flagellar-antiserum which has been developed against motile bacteria. The flagellar-antiserum is more sensitive than the somatic-antiserum to dilution than somatic

agglutination. Both the flagellar and somatic antigens are not entirely specific, since each may be shared by several distinct organisms. Thus the somatic antigen of the typhoid bacillus is the same as that of *B. enteritidis*, *B. pullorum*, etc., while the flagellar antigen of *B. typhosus* occurs also in the Stanley type of *Salmonella* in one phase. Again, many organisms of this group exist in specific and non-specific phases; the former may be characterised by flagellar antigen peculiar to a single species, e.g. *B. paratyphosus B*, while the antigens of the non-specific phase are common to most species. Certain

members of the group, *e g* the typhoid bacillus, the paratyphoid A bacillus, etc., exist only in the specific phase; others, *e g* the Berlin variety of the Thompson type of *Salmonella*, show only the non-specific phase. Again, in the Flexner group of dysentery bacilli the specific somatic antigens characteristic of the different types tend sooner or later to be lost on continued cultivation, these being replaced by group antigens. In addition to the somatic and flagellar agglutinogens, one has also to add that in the rough and smooth variants of certain organisms the former differ from one another. Thus the receptor analysis of bacteria, especially when motile, comes to be a complicated matter as is well exemplified in the salmonella group. The cholera vibrio also illustrates the fact that non-protein carbohydrate constituents may be the agglutinogens responsible for the serological characters of bacteria, according to Bruce White there may be present in the S-form four such components, the suppression of one or more of these being associated with the formation of variant types of the organism. Also, it has been suggested that various antigens are disposed at different depths in the bacterial body. Therefore, clearly the conditions for making a diagnosis by means of agglutinins must be worked out in the case of each organism. The evidence goes to show that the somatic agglutinins are the same as the bactericidal and complement-fixing immune-bodies (Felix), at any rate they are more closely related to these than are the flagellar agglutinins and accordingly constitute a better index of true immunity.

absorption (*vide infra*), by removal of the antibodies to the flagellar and somatic antigens from an antiserum containing a mixture of all three antibodies, a preparation can be obtained which exhibits anti-V₁ action alone. The agglutinated organisms form fine granules. Subsequently a strain of *B*

V₁ agglutinin, also it promotes their phagocytosis, and it confers passive immunity on mice against inoculation with virulent typhoid bacilli. It is, however, not the sole protective antibody in anti-typhoid serum, since it is inferior to an anti-somatic serum in protecting against infection with organisms of low virulence or the toxic effects of killed cultures. The V₁ antigen, which is highly specific for the typhoid bacillus (although found in certain other organisms, *e g B paratyphosus C*), is present in recently isolated cultures but tends to disappear unless the organisms grow under particular conditions, *e g* not below 25° C or above 40° C. It is demonstrated in its most characteristic form in living organisms and resists treatment with alcohol, but is readily damaged or destroyed by various agencies, *e g* phenol, formaldehyde, or heat, although it may partially resist 100° C. After prolonged subculturing in artificial media the V₁ antigen may still be formed and it has been found in non-virulent rough variants. A similar antigen has been detected in salmonella bacilli.

The facts outlined above have an important bearing on the selection of

strains of organisms for

tion. In or

organisms r

acute in developing protective antibodies, as in the case of the salmonella group, the somatic antigens of the smooth type and also the Vi antigen; these conditions are best fulfilled by recently isolated virulent cultures. Further, care must be taken that in the process of sterilising the vaccine its antigenicity is not

There is evidence that

not affect

impair its

Estimation of Agglutinins. The essential process is the bringing of diluted serum into contact with the bacteria uniformly dispersed in a fluid. The object is to ascertain the highest dilution of the serum which will effect agglutination, accordingly, a series of dilutions is employed. The organisms are used either as a broth culture or a suspension made by adding a small portion of an agar culture to broth or 0.85 per cent. solution of sodium chloride. Agglutination is increased in rate by a rise of temperature from 0° to over 30° C. If the result is watched *under the microscope* in a hanging drop preparation the occurrence of the phenomenon is shown by the aggregation of the bacteria into clumps, and if the organism is motile this change is preceded or accompanied by more or less complete loss of motility. At first the clumps are small, when larger they can be seen at a low magnification and finally they become visible to the naked eye. If the mixture is placed in a small test-tube, sedimentation is shown by the formation of a deposit, the fluid above being clear. Two points should be attended to: (a) controls should always be made with normal serum—to exclude the action of natural agglutinins, and with the bacterial suspension alone—to exclude auto-agglutination, and (b) the serum to be tested should never be brought in the undiluted condition into contact with the bacteria. For the *naked eye test*, equal volumes of serum dilutions and of bacterial suspension are placed in agglutination tubes (or narrow test-tubes). These are kept in the upright position at room temperature for twenty-four hours, or at 37° C. for four hours, or at 50° to 55° C. in a water bath.

The highest dilution of serum which produces agglutination is 1 : 8,000, this is expressed by saying that 8,000 is the 'titre' of the serum. The results are best read with the naked eye by holding the tubes against a dark background with a bright light in front of the observer. This method of estimation is much preferable to the more accurate Flag

Flocculation on a slide. This method is for preliminary observations, e.g. for testing suspicious colonies from plate cultures of faeces in order to determine which should be submitted to further examination. Drops of a low dilution of agglutinating serum (e.g. 1 : 100 of an antiserum with a titre of 5,000) are placed on a slide and a loopful of organisms from each colony is rubbed up with the needle in successive drops, so as to yield a very dense turbidity, then the slide is rocked vigorously for a minute or so. If flocculation occurs, granularity obvious to the naked eye develops.

Standard agglutinable cultures. Since different cultures of the same organisms (and even different subcultures of a single strain) may differ in their agglutinability by the same antiserum, Dreyer devised a method of standardising such cultures. The organism (e.g. *B. typhosus*) is grown for twenty-four hours at 37° C. in ordinary broth. At the end of this there

is added to it 0.1 per cent. of commercial formalin; it is shaken and kept at about 2° C. in the dark. At the end of three or four days the culture will be found to be sterile and will keep practically indefinitely. Such killed cultures are very suitable for sedimentation tests. When a new specimen of culture is to be employed, it is tested for agglutination with varying dilutions of an antiserum, so as to determine the titre of the latter. Simultaneously, the titre of the same antiserum is determined when used along with the standard culture¹. If the two titres differ, then the former divided by the latter yields a factor which must be employed to divide the titres of all sera tested with the new culture in order to make them comparable with results obtained with the standard culture. In this way successive batches of the agglutinable culture are compared and the factor for each is determined. Accordingly, where the agglutinability of a batch of bacterial suspension is not standard, the 'standard' titre is gained by dividing the actual titre (obtained as already described) by the suspension factor which is supplied.

Flagellar and somatic agglutinins It may be important to determine which types of agglutinin are present in the serum, and for this purpose 'qualitative receptor analysis' is carried out (Felix), e.g. in certain cases of typhoid and salmonella infections. Strains of organisms rich in the respective antigens are selected and grown on appropriate media, e.g. to promote the formation of flagellar (H) antigen the agar medium should be prepared from fresh meat (not meat extract), react neutral to litmus, have abundant water of condensation and be sterilised at 100° C. For detection of flagellar agglutinin, suspensions of living organisms are best, but failing these, preserved suspensions prepared with phenol or formalin may be used.

For somatic (O) agglutinin, cultures of non-flagellate variants of motile organisms are used, or motile cultures treated with alcohol or boiled. The titre of the serum may then be determined in the usual way by the naked eye method. In the case of living suspensions the results are read with a $\times 10$ magnifier after two hours at 37° C., and again after sixteen to eighteen hours further at room temperature, since small flake agglutination occurs slowly. With alcohol-treated suspensions twenty-four hours at 50° to 55° C. should be allowed.

V₁ agglutinins Owing to the very finely granular character of the sediment which forms, it is advisable to use wide tubes ($\frac{1}{2}$ in diameter). A suspension of a suitable culture is mixed in the usual way with dilutions of serum. The mixtures are kept for two hours at 37° C. and thereafter for twenty-two hours at a uniform room temperature (convection currents must be avoided). A positive result is shown by the formation of small clumps, visible to the naked eye, which occupy from a half to the whole of the base of the tube, the supernatant fluid becoming clear. In the control tube without serum or where the result is negative the organisms are partially deposited, forming a small circular layer with a sharply defined margin.

Practical Applications of Agglutination. The agglutinating property of the blood serum for a particular organism may be increased as a result of infection with that organism. This increase usually becomes apparent after the infection has lasted for about a week, but may be delayed or even fail to occur if the severity of the resulting disease damages the reacting power of the patient, also the increase in agglutinins may persist long after clinical recovery. Therefore in a case of suspected infection the finding of increased agglutinins for the organism is of value in *diagnosis*. It must be borne in mind, however, that the same result follows vaccination with suitable killed

¹ These agglutinable cultures are supplied by the Standards Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

cultures Further, the development of agglutinins may occur again in a later infection due to some other organism ('anamnestic reaction'). Felix has found in the case of the typhoid-paratyphoid group that it is the flagellar (H) agglutinins that are affected in this way. Thus in a patient at some time previously inoculated with a vaccine such as T.A.B., a reappearance of the corresponding agglutinins may result from a febrile attack due to some other infection. On the other hand, the somatic (O) and Vi agglutinins are not liable to this non-specific stimulation; accordingly, when they are present they have a diagnostic value. Again, infection with one organism may stimulate the production of agglutinins for another, e.g. for *B. proteus* in typhus fever. This non-specific stimulation is manifestly of importance in diagnosis. For these reasons the diagnostic significance of a positive agglutination reaction with a patient's serum is limited. In suspicious cases if the first test is negative or only weakly positive a further specimen of the serum obtained seven to ten days later should be examined; an increase in agglutinins ('rising titre') is of diagnostic significance, especially if the fallacies mentioned above can be excluded.

In addition to its value in the diagnosis of infections, the agglutination reaction is of great service in the differentiation of bacteria; in this case the agglutinability of an unknown bacterium is tested by a known antiserum. (For this purpose artificial antisera of high titre must always be employed; these are generally derived from rabbits—human sera from cases of natural infection should never be used.) If the unknown organism is agglutinated by the antiserum in the same dilution as the homologous organism (i.e. to titre, or even to half titre), this is evidence of their identity. One must recognise, however, that occasionally different pathogenic organisms react in the same way to an agglutinating serum and cannot be differentiated by this method. For example, in the three following instances, *Gonococcus* and *Meningococcus*, *B. melitensis* and *B. abortus*, and some strains of *B. mallei* and *B. whittmori*, it is recognised that the two organisms usually cannot be distinguished by direct agglutination. In the salmonella group also, different members may be agglutinated to the same titre as the homologous organism by an antiserum. In such cases of co-agglutination differentiation of the cultures can be effected by the method of agglutinin absorption—Castellani's reaction (*vide infra*).

Agglutinin Absorption. The principle is that the homologous organism will remove from an antiserum the agglutinins both for itself and for the heterologous organism, whereas the latter will remove from it only the group-agglutinins. Accordingly, if an antiserum to the known culture A agglutinates culture X, then tests made with the following results indicate that X is a heterologous organism.

Anti-A Serum absorbed with	Agglutinating Action of the Treated Serum on	
	Culture A	Culture X
Culture A	nil	nil
Culture X	practically unchanged	nil

On the other hand, if absorption of anti-A serum with culture X removes the agglutinating action for both A and X, then these two organisms may be identical. Their identity is confirmed if an Anti-X serum on absorption with culture A loses its agglutinating power for both A and X. In carrying out such tests attention must be paid both to the somatic antigens (whether smooth or rough) and to the flagellar antigens (whether in the specific or the group phase).

The method of 'absorbing' an antiserum consists in adding to a suitable dilution of the serum (*e.g.* a serum whose titre was 5,000 would be diluted ten times) an equal volume of a dense suspension of the organisms (one agar slope

and its agglutinating properties studied on the other members of the bacterial group and also, by way of control, on the organism used for absorbing it, in order to make sure that all agglutinins for the latter have been removed (If the control test shows that agglutinins for the absorbing organism are still present in the treated serum, absorption should be repeated with a further quantity of the same culture)

In this way the identity or otherwise of the flagellar or somatic agglutinogens may be established. Identification of an organism can thus be made under certain conditions, and related species can be distinguished by the absorption method. It has been supposed that each species of organisms contains a 'mosaic' of antigens and hence that the group reaction is due to certain antigens being common to several species. This explanation is questioned, however, by Burnet

When certain bacteria—*Shigella flexneri*—are grown in broth their morphological characteristics (threads or chains which are not observed in ordinary conditions)

Bor. recurrentis are mixed with the corresponding antibody and bacteria are added the latter adhere to the spirochaetes

Precipitins. It was shown by Kraus and by Nicolle that if an old bacterial culture be filtered through porcelain the addition of some of the corresponding antiserum produces a granular precipitate (The appearance of this deposit is hastened and intensified if minute inorganic particles or heterologous bacteria are added to the mixture) For the reaction it is necessary to have constituents of the bacteria in solution, and this may be attained by various methods, *e.g.* (a) by using an old broth culture in which the bacterial constituents have gone into solution, as in a culture which has been kept in the incubator for several weeks, the filtrate will then contain the reacting substance or precipitinogen (b) the growth from a recent agar or broth culture is suspended in normal salt solution, the mixture is made feebly alkaline with soda solution or acid with weak hydrochloric acid and boiled for a few minutes, the mixture is then neutralised, when a precipitate forms, and is filtered through filter paper, the filtrate contains the precipitinogen (c) cultures are extracted by the various methods used for obtaining soluble antigens or haptens. A very delicate method of demonstrating the phenomenon is the ring test, it consists in floating varying dilutions of the bacterial extract on to the surface of a fixed amount of antiserum in a small tube. The occurrence of precipitation is shown by the appearance of a ring of opacity at the junction of the fluids

In eliciting the agglutination and precipitation reactions there is in general a noteworthy difference in procedure, in the former a minute amount of antiserum acts on a large quantity of the organisms, whereas precipitation is obtained when an excess of antiserum is mixed with a small amount of antigen. This precipitin reaction has now been observed

in many bacterial infections when the patient's serum is added to the corresponding bacterial filtrate; conversely, the presence of soluble constituents of the causal organism has been shown by adding the corresponding antiserum to urine in cases of pneumococcal pneumonia or to the cerebro-spinal fluid in meningococcal meningitis, etc. Thus the reaction has been applied as a means of diagnosis. It is, however, more restricted in its application than the agglutination method. In the diagnosis of anthrax infections the presence of antigens of the anthrax bacillus in extracts of an animal's organs may be demonstrated by precipitation. The reaction may also be given by haptens along with the homologous antisera, for example, the specific soluble substance of pneumococci, the carbohydrate-containing hapten extracted from tubercle bacilli by Laidlaw and Dudley, and in the case of streptococci the precipitinogen extracted from the various groups by Lancefield's method.

The identity of the antibody which brings about precipitation with that which causes agglutination has been demonstrated by Heidelberger *et al.* in the case of anti-pneumococcus serum. A solution of pneumococcal specific soluble substance was precipitated by the homologous antiserum. Then the precipitate was treated with a solution of the antiserum. The precipitate then dissociated and the antibody A solution of the same type caused them to agglutinate.

Serum Precipitins. This subject does not strictly belong to bacteriology, but the general phenomena are so closely allied to those just described that some reference may be made to it. When the serum of an animal is injected in repeated doses into another

doses of 3 to 4 c.c. of the serum may be injected intraperitoneally at intervals of four to five days, a precipitin usually appearing at the end of about three weeks. Dean obtained the best precipitins by giving six to eight injections each of 2 c.c. of serum at intervals of five days—the first few being intravenous and the rest intraperitoneal—and then after a rest period of three to six months a second series of injections. The reaction, which is a very delicate one, is conveniently observed by adding a given amount of the antiserum, say 0.05 c.c., to varying amounts of the homologous serum 0.1, 0.01, etc. c.c., in a series of small test-tubes, the volume being made up with salt solution to 1 c.c. In this way a definite reaction may be observed with 0.0001 c.c. of the homologous serum or less. An even more delicate reaction is obtained when the solution of antigen is placed with a pipette on the surface of the solution of precipitin, a layer of cloudiness then forming at their junction—the so-called 'ring' test. If the antiserum be heated to a temperature of 75° C. for some time it acquires inhibitory properties, so that when added to a mixture of serum and antiserum which would otherwise give a precipitate, this no longer occurs. Some observers consider that this is due to the presence of 'precipitoid' in the heated antiserum, but the observations of Welsh and Chapman show that this view is not in accordance with the facts, and indicate that the inhibition is related to a specific solvent action which the heated antiserum has on the precipitate. They have also shown that the main mass of the precipitate is furnished by the antiserum (precipitin), and not as had been supposed by others, by the antigen. The precipitin reaction is specific in the sense explained above. It is always marked towards the serum of the species used in the immunisation; but while this is so, there may also be a slight reaction towards animals of allied species. An antiserum may also be employed as a means of differentiating human from other bloods. For critical quantitative determinations of either antigen or antibody Dean employs a method for determining optimal proportions, i.e. those in which the most rapid formation of particles occurs. First, a rough test is performed in which the following antiserum dilutions—1:5, 1:10, 1:20, 1:40—are each mixed with a series of eleven dilutions of antigen ranging from 1:10 to 1:10,000. The concentration of antiserum which yields precipita-

be employed as a means of differentiating human from other bloods. For critical quantitative determinations of either antigen or antibody Dean employs a method for determining optimal proportions, i.e. those in which the most rapid formation of particles occurs. First, a rough test is performed in which the following antiserum dilutions—1:5, 1:10, 1:20, 1:40—are each mixed with a series of eleven dilutions of antigen ranging from 1:10 to 1:10,000. The concentration of antiserum which yields precipita-

tion in a convenient time is noted and also the dilution of antigen with which this occurs. Then by mixing 1 c.c. amounts of this concentration of antiserum with a series of 1 c.c. volumes of varying dilutions of the antigen, each differing by 0.00025 c.c. of serum, the optimum is ascertained.

Another important phenomenon, the reaction of complement fixation, is produced by the combination of the two substances in the serum (antigen) and antiserum (anti-

complement becomes absorbed, as may be shown by subsequently adding a test amount of sensitised red blood corpuscles. This fixation phenomenon is an even more delicate reaction than the precipitin test, it being often possible to demonstrate by its use from a tenth to a hundredth of the smallest amount of serum which will give a perceptible precipitate; it also is specific within the same limits.

Mechanism of Agglutination and Precipitation. The chemical and physical changes on can be said to be fully understood. The phenomenon. (a) the combination of (agglutinin), and (b) the actual clumping of the bacteria. For the occurrence of the latter an essential factor is a suitable salt content. Bordet showed that when the bacterial suspension and the agglutinin are made salt-free by dialysis, agglutination does not take place on their being mixed, nevertheless the agglutinin is fixed by the bacteria. On the addition of an electrolyte such as sodium chloride in small amount agglutination occurs. Further, if subsequently the clumps of agglutinated bacteria are again freed from salt by washing in distilled water they become resolved, but on the addition of sodium chloride they are formed again. There is an optimum salt-concentration for agglutination. It is also to be noted that many strains of bacteria, e.g. the rough forms of the coli-typhoid group, agglutinate in salt solutions without the

cause agglutination, e.g. the typhoid bacillus in the smooth form when it possesses Vi antigen is agglutinated by tryptaflavin.

A further important point is that the occurrence of agglutination after the combination of agglutinin and agglutininogen depends on the physical condition of each, as well as on the presence of electrolytes. For example, when the bacteria are heated at a temperature of 65° C. for some time, they may lose the faculty of being agglutinated while they may still retain the property of combining with or binding agglutinin. Dreyer and Jex-Blake have observed the remarkable fact that on being heated to a still higher temperature the organisms may once more become agglutinable. Alterations in the antibody may also produce differences in the agglutination effect. Thus antisera which have been heated to 60° to 70° C. undergo a modification, so that a zone effect occurs, i.e. the higher concentrations of serum fail to cause agglutination, while lower strengths, in the neighbourhood of the titre of the unheated serum, are still effective. It appears that in this case an 'agglutinoid' is formed by the heating, which has a higher combining affinity for the organisms than that of the unmodified agglutinin, but which does not produce agglutination. Antisera which have not been subjected to any special treatment may contain an agglutinoid-like antibody which combines with the corresponding antigen and prevents the usual action of an agglutinating antiserum added subsequently. Such 'blocking' antibody has been met with for the Rh-antigens of human erythrocytes (Race). Further aspects of the zone phenomenon are discussed below.

It is agreed that agglutination and precipitation are closely allied

phenomena and present certain similarities to well-known reactions exhibited by colloids. In both there is a reduction in the dispersion of colloid particles; in agglutination, these are the bacteria in suspension, and in precipitation the colloidal particles of the bacterial extract or the protein in solution. It is recognised that the chief force which keeps particles or molecules apart in a fluid is the like electric charge which they bear, while surface tension is the cohesive force which tends to draw them together. Experiments on the electrical state of *B. typhosus* when suspended in weak neutral saline solution have shown that the organisms carry a negative charge; a reduction of this charge below a certain critical level will lead to their adhesion. A cation such as sodium reduces the surface charge only when in relatively high concentrations and so does not agglutinate the bacilli unless they are combined with agglutinin. Bacteria sensitised by antibody behave like particles of denaturated globulin; and this supports the view that they are more or less coated with the antibody. Further, the complex, antigen plus antibody, has

hydrophobic. The hydrophilic group of the antibody is believed to combine with the organism, thus causing the hydrophobic group to point towards the watery medium in which the organisms are suspended. It is therefore the latter group which imparts its properties to the sensitised organisms. Another striking resemblance to colloidal interaction is seen in the zone phenomenon. For example, when the amount of precipitin is kept constant in a series of tubes and gradually increasing amounts of precipitinogen (antigen) are added, the resulting precipitate increases up to a point, but beyond that point increased amounts of antigen cause diminution of the phenomenon is also modification phenomenon is

seen when, for instance, increasing amounts of a colloidal solution of iron hydroxide are added to a mastic emulsion. This phenomenon has been explained as being due to alteration of the electric charge of the particles of the one colloid by the added particles of the other, till the charge is brought to zero, there then occurs the maximum precipitate. Further addition leads to reversal of the charge, and thus once more to dispersion of the particles.

Even if it is the case, as seems certain, that the physical phenomena of agglutination and precipitation correspond with those in the interactions of other colloids, it must be borne in mind that the essential factor is the specific combination of antigen and antibody—of agglutinin and agglutinin, of precipitinogen and precipitin; and to this there appears to be no analogy in colloidal reactions. Interesting results have been recorded by Topley and others, which suggest that in addition to the factors mentioned there is some specific affinity between antibody-laden organisms belonging to the same species. This view is supported by the observation that when mixtures of two species of organisms, e.g. pneumococci and typhoid bacilli, are agglutinated by a mixture of the corresponding antisera, the clumps which form are composed practically wholly of the one kind of organism or the other. Accordingly, in Marrack's view, the antibody would be regarded as having several combining groups and thereby linking up those organisms for which it has a specific affinity into a sort of lattice-work. (The experimental results, however, have not been wholly confirmed.) On the same principle the phenomenon of precipitation would result from the aggregation of molecules

of antibody with the precipitinogen. These aggregates could obviously vary in the proportions of the two constituents, and this agrees with chemical analyses of precipitates (Heidelberger). Pijper studied by cinematographic methods the agglutination of typhoid bacilli and found that an anti-somatic serum led to the bacilli attracting each other. In the case of an anti-flagellar serum, the flagellar structures became coated with a sheath of granules, thereby being stiffened, finally a tangle of these stiff forms occurred mechanically from chance contacts, there being no evidence of attraction.

(d) **Antiaggressins.** In the case of certain organisms such as the anthrax bacillus, the serum of actively immunised animals does not manifest *in vitro* any of the properties of antibodies described above; nevertheless it confers passive immunity, but it is not readily deprived of its protective action by treatment with the homologous organisms in the test-tube. Bail has attributed the action of such antisera to antiaggressins. Experimental evidence in support of this view has been furnished by Pettersson, who has shown that if anthrax aggressin and virulent anthrax bacilli be mixed with normal rabbit serum in one case and in the other with anti-anthrax immune serum, and the mixtures are then injected subcutaneously into a normal rabbit, different results follow. At the site of injection of the mixture containing immune serum leucocytes collect in abundance, but where the mixture containing normal serum was injected leucocytes are absent. Accordingly, the antiserum appears to inhibit the action of aggressin in preventing accumulation of leucocytes. Another example of anti-aggressin action is the specific effect of anti-pneumococcal type serum in combining with the capsular polysaccharide of the corresponding type which, although non-toxic by itself, has aggressin functions.

UNITY OR PLURALITY OF ANTIBODIES

In the above account different antibodies have been mentioned, but, as already stated, the names used merely indicate the possession of different properties by antisera as revealed by interaction with antigens. There has been, and still is, dispute as to whether these properties correspond with different substances or whether all the properties are borne by one—the 'pluralistic' and 'unitarian' views respectively. In the first place, we may say that there is no justification for inferring the existence of a particular substance corresponding to each reaction. In all cases the union of the antibody causes some change in the bacterial antigen, and this may result in various effects according to the method of test. Thus we may have agglutination, precipitation, increased adhesiveness and opsonic action, or fixation of complement, which in its turn may lead to the death of the organism or to its being opsonised, or, lastly, to no observable effect. All those effects may possibly be produced by the same antibody, and the close relation between both agglutination and precipitation and complement fixation has already been shown. Frequently the obvious manifestations of the interaction between antigen and antibody depend on the presence of some third agent which, as it were, acts as an indicator. Thus, Hartley found that antitoxic serum from did not flocculate the corresponding toxic effects as tested by injection in er hand, an anti-typhoid serum which had been similarly extracted still agglutinated typhoid bacilli in as high dilution as the untreated serum, while in the latter reaction the essential part played by electrolytes in flocculating the agglutinin-laden bacteria has already been mentioned.

It does not follow from what has been stated above, however, that all antibody molecules are similar in the sense that each is capable of bringing about in a proportionate degree the various reactions which have been described. The study of antisera in the course of development shows that at different stages there is considerable variation in the extent to which the different antibody reactions are produced. Also, it has been found in the case of hæmolytic serum that the immune-body shows qualitative changes at different stages. It does not seem possible to reconcile such results with the idea of a single antibody. Therefore, it would appear that antibodies may differ in other respects than in their combining groups; that is, they are not uniform in their reacting properties. On the other hand, various reacting properties may be held by the same antibody. A further point which must be considered in regard to the action of an antiserum, *e.g.* to an organism, is that the latter may contain a multiplicity of antigens, each of which may lead to the development of a corresponding antibody. The flagellar, somatic, and V_1 antigens of the typhoid bacillus illustrate this. According to Felix it is the somatic antibody which is most active in leading to fixation of complement. Again, different animal species may respond to the injection of the same antigen by the production of antibodies which are not identical in their properties.

THE NATURE AND SOURCE OF ANTIBODIES

The earlier work on these questions was concerned mainly with antitoxins, and various theories were put forward. One of the first views to be advanced was that antitoxin molecules represented toxin molecules which had been in some way modified by the cells of the body; but it was soon established that this view could not be maintained. It was found that the amount of antitoxin produced by an animal may be many times greater than the equivalent of toxin injected, and, further, that when an animal with a practically stationary antitoxin level in its blood, is subjected to repeated bleedings, the amount of antitoxin in the blood may some time afterwards be greater than it was immediately before bleeding, even although no additional toxin is introduced. Similarly, if the blood of an actively immunised animal containing antibodies is removed and replaced by the blood of a normal animal, the antibodies are rapidly restored. The latter circumstances show that antitoxin is formed by the cells of the body. The view that the antigen does not enter into the composition of the antibody has been confirmed in other ways. For instance, in the case of an antigen composed of the arsenical drug atoxyl diazotised and combined with foreign protein, the resulting antiserum derived from a rabbit, when tested in a considerable quantity contained no demonstrable amount of arsenic (Berger and Erlenmeyer). This being so, it remains to be determined whether the antibody is a normal constituent of the cells which is formed in increased quantity or whether it is a new product. The presence of representatives of a great multiplicity of antibodies in normal sera is a highly significant circumstance, as these are undoubtedly the products of cellular activity, and in all probability molecules of corresponding nature occur as constituents of cells; an increased formation and setting free of these may therefore explain the production of antibodies in active immunity. It should be noted that many of those natural antibodies act on antigens with which the animal's tissues cannot possibly have come into previous contact. But do all antibodies have normal representatives? If not, then the molecules of antigen must by a sort of impress alter the configuration of molecules in the cells so that they function as antibodies.

Regarding such an occurrence, however, we know nothing. As to the manner in which the antigens influence the cells, there is evidence that in the living body bacterial toxins enter into combination with, or, as it is often expressed, are fixed by the tissues—presumably by means of certain combining affinities. This has been shown by the experiments of Domitz and of Heymans with tetanus toxin. In such cases there is no evidence as to where the toxin is fixed beyond that supplied by the occurrence of symptoms. We may note, however, that it is not a serious objection that in certain animals other tissues than that of the central nervous system can combine with tetanus toxin—this might take place with or without resulting symptoms. It is, moreover, evident that the molecules in the cells which unite with toxin may, when set free, act as antitoxin by neutralising the toxin and thus preventing its combination with the cells.

THE NATURE OF ANTIBODIES From filtration experiments Brodie, and Martin and Cherry, deduced that the antitoxins were large molecules. It had previously been found that the precipitate of globulins obtained by adding appropriate concentrations of magnesium or ammonium sulphate to anti-diphtheria serum, anti-tetanus serum, or the milk of immunised animals, contained practically all the antitoxin. Further, it was determined by Ide and Lemaire, Pick, and others that the antitoxin was not uniformly distributed amongst the various globulin fractions which may be obtained from sera, but that it was largely restricted to a particular component—the pseudoglobulin in the case of diphtheria antitoxin from the horse. Hiss and Atkinson, and also Ledingham found that the percentage amount of globulin precipitate from the serum of the horse increased after it was treated in the usual way for the production of antitoxin. This increase, however, does not appear to be an essential accompaniment of antibody formation, further, the actual antibody has been estimated to account for only a part of such increase. Similar results have been obtained with regard to the other classes of antibodies. They are in general found along with the globulins, though the fraction with which they are chiefly associated varies in different cases; and the possibility must be considered that they may merely be adsorbed by the globulins. It may be mentioned in this connection that according to Huntoon *et al.*, antibodies separated from their combination with pneumococci did not correspond in their reactions with serum proteins, for example, they were not affected by the same precipitants as the globulins by saline solutions. But, really globulins, been sought by

two methods: (1) by ascertaining their rate of sedimentation in relation to that of the various constituents of normal serum in Svedberg's ultracentrifuge and (2) by determining their rate of migration in an electric field with the electrophoresis apparatus of Tiselius.

It has been shown that the rabbit produces serum globulin in molecular weight, and following intravenous injections of pneumococci the resulting antibody is heavier than normal globulin. Electrophoretic studies have indicated that of the three globulin components of normal rabbit or horse serum which can be distinguished, namely, alpha, beta, and gamma, the last is associated with antibodies in the rabbit, and the same is true in man. In the horse a new component may occur intermediate between beta and gamma globulin, which seems to consist wholly of antibody, since it is removed by absorption with a homologous antigen, or there may be antitoxins associated with both beta and gamma globulins (Kekewik *et al.*).

Methods of purification. The association of antibodies with certain serum globulins affords a valuable basis for practical methods of concentrating and purifying antitoxins and other antibodies. The following procedures have been described by Pope in the case of anti-diphtheria serum from the horse for producing a 'refined' antitoxin. (1) The pseudoglobulin fraction (separated by 30 to 50 per cent. saturation with ammonium sulphate) which contains practically all the antitoxin, is subjected to very slight treatment with proteolytic ferments, *e.g.* fibrinolysin (extracted from fibrin with acetic acid or by autolysis in 0.2 per cent. tricresol solution) or pepsin, short of hydrolysis or digestion as ordinarily understood; then the non-antitoxic part is removed by critical heating (58° C. for thirty minutes) in 15 per cent. ammonium sulphate solution at an acid reaction, thus leaving the antitoxic moiety of the pseudoglobulin in solution. A modification of this method is applicable to plasma. (2) The crude pseudoglobulin fraction is denatured by critical heating in acid solution until it is rendered insoluble. Then by the action of pepsin on the coagulum a soluble portion is split off which contains much of the antitoxin; this is salted out. Another procedure which has been employed for isolating antibodies consists in combining a particulate antigen, *e.g.* bacteria, with the corresponding antibody, or in the case of an antigen in solution, such as a toxin, obtaining a precipitate with the antitoxin. Material remaining in solution is then removed by centrifuging, and the residue is washed and finally dissociated by altering the reaction or the salt content of the medium or by electrical dialysis. It has been stated by Northrop that digestion of a diphtheria toxin-antitoxin precipitate with trypsin and subsequent fractional salting out resulted in removal of the toxin and the isolation of a very pure antitoxic protein.

Regarding the nature of the complex which is formed by antigen and antibody, little is known. As has been seen, provided the action of complement is excluded the combination is almost always a reversible one. It has been supposed that the antibody globulin forms a layer covering the antigen and that a regular arrangement of the molecules takes place on the analogy of that which is supposed to occur at surfaces of separation under the influence of polar forces, as in a condensed film of palmitic acid floating on water, where the carboxyl groups are all orientated towards the water and the hydrocarbon chains towards the air. Owing to the small amount of antiserum which may be effective in bringing about an immunity reaction, *e.g.* agglutination, it is possible that the antibody need not completely coat the surface of the antigen, but simply becomes attached at particular places. In this way the antibody would serve to link the molecules of the antigen together and so produce a lattice (*vide supra*). The actual amount of antibody contained in an antiserum has been measured chemically in the following way (Heidelberg *et al.*) An anti-pneumococcus serum is precipitated with the corresponding specific carbohydrate, which contains no nitrogen. Then the precipitate is washed and its nitrogen content ascertained; this determines the antibody content of the antiserum.

SOURCE OF ANTIBODIES. The local production of antibodies has been demonstrated in several instances. In the case of rabbits undergoing immunisation with killed *V. cholerae*, Pfeiffer and Marx found a greater concentration of bactericidal antibody in the spleen, bone marrow, and lymph glands than in the blood. Similar observations have been made with regard to agglutinins; and it has been shown that after intradermal injections of killed organisms the lymph glands draining the area actually form the antibody and do not merely accumulate it (McMaster and Hudack *et al.*) After the injection of foreign protein into the anterior chamber of the eye,

precipitin has been detected in the aqueous humor of that eye before it was present in the blood (Dungern). *Hæmolytic immune-body* is stated to have been formed after a few days in tissue cultures of bone marrow and lymph gland, and *bactericidal immune-body* in that of splenic tissue, to which the respective antigens have been added. Experimental investigations aimed at discovering the chief sites of origin of antibodies have yielded comparatively few facts. Thus Buttle has found in the case of rabbits immunised with diphtheria toxoid that neither the blood, liver and spleen, nor the skin is predominantly responsible for the production of antitoxin. In view of the phagocytic capacity of cells of the reticulo-endothelial system, it has been supposed that they produce the antibodies. Sabin injected parenterally a finely particulate suspension of a dye-protein antigen and found that the occurrence of antibodies in the serum coincided with the disappearance of the dyed particles from the cells of the reticulo-endothelial system. At this time shedding of surface films from the monocytes and macrophages was observed when these cells were examined *in vitro*. It is inferred that the shed material contributes the antibody globulin to the blood. It appears likely that these cells are concerned in the production of antagonistic and protective substances, but no quite conclusive evidence for this has been obtained. The phenomenon of 'delayed response' on reinjection of toxoid (p 84) has led to the conclusion that the time rates of the immunity response of different tissues may vary. Attempts to demonstrate interference with antibody formation by 'blockade' of the reticulo-endothelial system have led to inconclusive results.

The incorporation of amino-acids of the food in antibody has been shown as follows. Actively immunised animals at the stage of declining antibody production were fed with amino-acids containing heavy nitrogen as a marker. The latter was afterwards detected in precipitates formed by adding the antibody to the soluble antigen or hapten (Schœnheimer *et al*).

Antibody, e.g. antitoxin, when present in the serum, occurs also in the lymph and various secretions and transudates, but is present in those in much less concentration than in the blood. Antitoxin is present in the milk of actively immunised mothers, and a certain degree of immunity can be conferred on new-born animals by feeding them with such milk, as has been shown by Ehrlich, Klemperer, and others. Bulloch also found in the case of hæmolytic sera that the antibody is transmitted from the mother to the offspring in the milk. In the case of goats and cattle it is the colostrum which is specially rich in antibodies. Klemperer found traces of antitoxin in the yolk of eggs of hens whose serum contained antitoxin.

MECHANISM OF ANTIBODY FORMATION Ehrlich's side-chain theory was an application of views regarding the assimilation of nutrition by cells based on structural conceptions of organic molecules. A molecule of protoplasm may be regarded as composed of a functional centre or atom-group along with a large number of side-chains consisting of atom-groups with combining affinity for nutritive substances of complex constitution. By means of the latter the living molecule is supposed to be built up in the process of nutrition, and hence the name *receptors* given to the combining apparatus. In considering the application of this idea to the facts of acquired immunity, it must be kept in view that in apparently every case the antibody enters into combination with its corresponding antigen. The dual constitution of toxins and kindred substances, as already described, is also of importance in this connection. When toxins are introduced into the tissues they are fixed, like nutritive substances, by their haptophore groups to the receptors of the

cell protoplasm, but they are unsuitable for purposes of nutrition. If they are in sufficiently large amount, the toxophore part of the toxin molecule produces disturbances of the protoplasm which are shown by symptoms of poisoning. If, however, they are in smaller quantity, as in the early stages of immunisation, fixation to the protoplasm occurs in the same way; and as the combination of receptors with toxin is supposed to be of firm nature, the receptors are lost for the functional purposes of the cell. The receptors thus lost become replaced by new ones, and when additional toxin molecules are introduced, these new receptors are used up in the same manner as before.

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where they constitute antitoxin molecules. Thus there are three factors in the process, namely (1) fixation of toxin, (2) over-production of receptors, (3) setting free of receptors produced in excess. Accordingly, identity is claimed for (a) those receptors which, as part of the cell protoplasm, anchor the toxin to the cell and so are essential to the occurrence of toxic phenomena, and (b) the antitoxin molecules, receptors in the free condition in the blood, which unite with the toxin, and thus prevent the toxin from combining with the cells and exerting a pathogenic action. From what has been stated it is manifest that any cell which fixes toxin is potentially a source of antitoxin, *e.g.* cells of the nervous system in the case of tetanus, though other tissue cells not seriously affected by toxin which they have fixed, may act in the same way.

Ehrlich's theory accords well with many of the known facts of active and passive immunity. In the case of antitoxins, we see that their formation should depend upon the combining affinity of the toxins for tissue cells and at the same time be independent of the poisonous properties; this accords with the immunising action of toxoids. It is to be noted, however, that it does not explain active immunity apart from the presence of antibodies in the serum. For example, an animal may be able to withstand a much larger amount of toxin than could be neutralised by the total amount of antitoxin in its serum, or may possess immunity when antibodies have disappeared from the blood. This might be explained by supposing a special looseness of the cell receptors so that the toxin-receptor combination became readily cast off; also it is known that the response to a secondary stimulus is greater both in rate and amount than that to the primary. The question arises, however, whether there may not be really an increased resistance of the cells to the toxic action—a genuine tissue immunity. An observation made by Meyer and Ransom is also difficult of explanation, according to the view that antitoxin is formed by the cells with which the toxin combines and on which it acts. They found that in an animal actively immunised against tetanus and with antitoxin beginning to appear in its blood, the injection of a single M L D of tetanus toxin into a peripheral nerve brought about tetanus with a fatal result. On the other hand, the injection of antitoxin into the sciatic nerve above the point of injection of toxin prevented the latter from reaching the cells of the cord. One can scarcely imagine an explanation of these facts if antitoxin molecules were in process of being shed off by the cells of the nervous system. Further, when the serum of an animal contains a large amount of antitoxin, how does the additional toxin injected reach the cells in order to influence them as we know it does? This also is difficult to understand, unless the toxin has a greater affinity for the receptors in the cells than for the free receptors (antitoxin) in the serum. A further difficulty is that in

general, antibodies cannot be recovered to any marked degree from the blood-free tissues of immunised animals; this, however, is not a serious objection, since the cells are secreting antibody and not necessarily accumulating it. On the other hand, the existence of sessile receptors is supported by observations in connection with anaphylaxis. The multiplicity of antibodies which may be developed, demands that a preformed receptor for each antigen should exist in the cellular protoplasm, this assumption is an unsatisfactory feature of the theory. One cardinal point which is not taken into account is that antigens, in respect of their foreign specificity, differ from the natural food-stuffs of cells, since such materials before absorption are normally deprived of specificity by the ferments in the alimentary tract. Accordingly, antibody formation represents a biological reaction on the part of the tissues when they come into contact with substances possessing such foreign specificity. A recent theory which embodies the essentials of Ehrlich's view, is that the injected antigen (or its derivative) on reaching the sites where globulin is being formed in the tissues, leads to some distortion of the products which is characteristic for the particular antigen (Breml and Haurowitz). Since antibodies tend to be formed for a considerable time after

producing mechanism persists. There is no clear evidence on which to decide between these possibilities. However, Burnet has drawn attention to the analogy between antibody formation and the development of adaptive enzymes by bacteria, which continue to be produced after contact of the substrate with the culture has ceased, so that descendants of the original cells still manufacture the enzymes. On this view antibodies to proteins are to be regarded as proteinases which have been developed as an adaptive response of the cells of the reticulo-endothelial system to the foreign protein with which they have come into contact. The experiments of Danielli *et al.* support their conclusion that the specificity of an antibody depends on the configuration of the protein molecule, since it was found that when an antiserum is spread out in a thin layer at a water-air or water-oil interface, denaturation consequent on unrolling of the molecules is associated with a loss of the capacity of the antibody to react specifically with the homologous

denaturation by uncurling of the ends of the protein molecule. The dye, methyl blue, was added and after the mixture had stood for half an hour it was dialysed. This led to gradual removal of the alkali and also some of the dye, and finally to a precipitate which contained proportionally much more dye than that present in precipitates from similar mixtures which were either rapidly neutralised or which contained no alkali. Accordingly, it was concluded that the globulin had acquired a new configuration complementary to that of the dye (antigen) molecule and then precipitated the latter, thus acting like an antibody. Another procedure was to mix a solution of globulin with an azo-dye derived from arsanilic acid, keep the mixture in slightly alkaline solution for two weeks at 57° C., *i.e.* below the denaturation temperature of the protein, then dialyse successively against saline, arsamate solution and again salt solution. The product, which was dye-free, acted as an antibody, causing precipitation and complement fixation along with the homologous antigen. By a procedure similar to the latter a type-specific antibody for pneumococci was also obtained.

Non-specific factors influencing antibody formation. Acting on the supposition that antitoxin formation is allied to a process of secretion, Salomonsen and Madsen tested the effect of pilocarpine and found that it had the property of producing a marked rise in the amount of antitoxin present in the blood of an animal. Walbum and others have also found that many metallic salts have a similar property of stimulating the formation of antibodies. They were tested on immunised animals after the antibody content had fallen to a steady level. Salts of different metals vary much in their action, but among the metals of the iron group manganese has the greatest effect; the most efficient salt of all was beryllium chloride. Mackie has shown that the normal antibody of the rabbit for sheep's corpuscles is increased by extensive bleedings and also by the administration of manganous chloride, beryllium chloride, colloidal manganese, salvarsan, etc. These non-specific factors are irregular in their action, however. The explanation of non-specific stimulation is quite obscure. Further aspects of non-specific formation of antibodies are discussed under natural immunity.

PASSIVE IMMUNITY : THERAPEUTIC USE OF ANTISERA

When antitoxins were discovered it was found that the serum of an actively immunised animal, if given in a sufficient dose parenterally to a susceptible individual, rendered the latter immune. The same principle holds in the case of other antibodies. The time taken for the antibody to reach the blood and tissues, and the concentration attained, depend on the route of injection. Such passive immunity, being due solely to the fixed amount of antibody introduced, is of much shorter duration than active immunity, where the antibody continues to be formed for a considerable time. Further, if, as is usually the case, the antibody is contained in serum of a species foreign to the animal injected, its disappearance is more rapid. With repeated injections the rate of elimination is increased.

Rate of absorption of antibody and concentration in the blood. By intravenous injection the maximum concentration is reached in the blood at once. Henderson Smith showed that after subcutaneous injection absorption is slow, the highest level of antibody in the blood not being reached for two to three days, also the blood level never approaches the maximum obtained after intravenous injection. Intramuscular injection is intermediate both as regards rate of absorption and level reached in the blood. Three days after an injection by any of these routes the antitoxin content of the blood is the same. The species of animal yielding the antibody also affects the results in the absence of any local irritation at the site of injection, e.g. after an intramuscular injection in man, pneumococcus antibody from the rabbit reaches the blood more quickly and in greater amount and disappears more slowly than that from the horse. Antibody practically fails to reach the cerebrospinal fluid from the blood stream, although it is absorbed in the reverse direction. Accordingly for the treatment of meningitis, serum should be injected intrathecally, as well as by other routes. But antibodies appear to pass the brain capillaries (Friedemann). In certain animals, such as the guinea-pig and rabbit, the actively or passively immunised mother transmits antibodies to the foetus through the placenta. The child *in utero* may be passively immunised by producing active or passive immunity in the mother. But recent work has indicated that antitoxin from the horse does not readily traverse the human placenta (Chesney).

Duration of Passive Immunity. After an intravenous injection of antiserum from a foreign species into a normal animal there is a rapid disappearance of the antibody from the blood, half being gone in six to twenty-four hours, this loss is probably due largely to diffusion into the tissues. Then a more gradual, progressive fall in the blood level occurs for six or seven days, by which time only a tenth of the original amount may be present. This is

followed by a phase of accelerated loss corresponding in time to the appearance in the blood of precipitins to the foreign protein. When there has been a previous immunity reaction ' ' ' ' of antibody is greatly accelerated the maximum concentration is and intensely as possible throughout the tissues (with the exception of the central nervous system) it should be administered intravenously; and this treatment may be reinforced by intramuscular injections. In order to maintain a state of passive immunity injections of antiserum should be repeated intramuscularly at weekly intervals, but with repeated doses the rate of destruction of antibody increases (Glenny *et al.*). If spaced injections of foreign serum are given the possibility of anaphylaxis must be remembered. When it is necessary to administer large doses of antibodies concentrated preparations of antisera are commonly used, which consist of the globulin fraction containing the antibodies. These serve the double purpose of reducing the volume injected and also of diminishing the amount of foreign protein, thereby lessening the tendency to serum-disease.

*Therapeutic use.*¹ The chief human diseases treated by *antitoxic sera* are diphtheria, tetanus, botulism, scarlet fever and other streptococcal toxæmias, staphylococcus infection, gas gangrene, snake-bite, and scorpion sting. The stage of the disease at which antitoxin therapy is started has an important influence on the result. It has been shown experimentally that when an animal has received a fatal dose of toxin, the earlier antitoxin is administered, the smaller the dose of antitoxin required to save its life. After a certain interval it is impossible to secure survival however large an amount be given. Thus may be the case even if antitoxin is administered before symptoms have appeared, since the antitoxin may be incapable of dissociating toxin fixed by susceptible cells, or the combined toxin may have already inflicted irreparable damage on these cells.

Antibacterial sera have chiefly been used in cerebro-spinal fever, pneumonia, dysentery, streptococcus infections, and plague. The methods of application and the general results will be dealt with under individual diseases. Antibacterial sera have not, on the whole, proved as efficacious therapeutic agents as antitoxic sera. A question of the highest importance is adequate dosage of the antibody. It has been found experimentally that an antibacterial serum in amounts short of the curative dose may produce little or no beneficial effect. Further, beyond a certain degree of infection no amount of serum will protect.

As has been shown above, antibacterial sera require for their bactericidal action a sufficiency of complement, as this disappears when a serum is kept, the unsatisfactory results with this class of sera may be due to a deficiency of complement. Or it may be, as Ehrlich suggested, that the complement naturally existing in human serum does not suit the immune-body in the

¹ The Therapeutic Substances Act, 1925, applies to the manufacture for sale of antisera in the same way as to vaccines. The Therapeutic Substances Regulations, 1931, 1935, and 1939, prescribe conditions with respect to which commercial therapeutic antisera must conform as regards their mode of preparation, sterility, freedom from abnormal toxicity, and content in solid matter, also in the case of diphtheria and tetanus antitoxins, gas gangrene antitoxins (*perfringens*, *oedematiens*, *vibrio septique*, and *histolyticus*), staphylococcus antitoxin, anti-dysentery serum (*Shiga* and others), and anti-pneumococcus serum (types I and II), the methods of standardising and the potency in relation to volume of serum or weight of dry products are prescribed. Provision is also made for the fact that on keeping antisera their potency gradually decreases, this loss being slow at temperatures near 0° C and much more rapid at 37° C. Antisera containing antiseptics should not be frozen, as concentration may occur in the protein material with consequent destruction of the antibodies.

antiserum and so is not taken up through the medium of the latter and brought into combination with the organisms. There is the further possibility that even though the complement should be taken up, the bactericidal action may not be sufficiently active to cause the death of the bacteria. In both cases it is clear that an extracellular bactericidal action cannot be produced by the particular immune-body in association with the complement of the animal concerned. There is no doubt that this question of differences in complements is a serious one, and that both combining affinity and cytotoxic action of complements must be considered in each case.

In such diseases as cerebro-spinal fever and pneumonia the opsonic mechanism of the infected individual may play a considerable part in successful resistance. The favourable effects following treatment with antisera may thus, in some cases, depend on an augmentation of the opsonic powers of the body.

COMBINED ACTIVE AND PASSIVE IMMUNISATION. The injection of antibodies along with the corresponding organisms or toxins (or toxoids) often does not prevent the latter from producing active immunity. Accordingly, it is becoming customary when prophylactic antiserum treatment is required, to inject at the same time the corresponding antigen. Thereby, in addition to passive immunity, the more lasting active immunity is developed.

INFECTION IMMUNITY : TISSUE IMMUNITY

In some infections manifestations of immunity are met with which cannot be explained satisfactorily by the mechanisms so far considered. For instance, after an individual has been infected with *Tr. pallidum* the skin soon ceases to respond to a further inoculation with the spirochaetes at a fresh site by the development of another chancre. This resistance to 'super-infection' persists so long as the body still harbours the spirochaetes, but if these are destroyed as the result of administering chemotherapeutic agents, the susceptibility of the skin to reinoculation soon returns. It appears therefore that this form of immunity lasts only so long as the infection persists. Accordingly, this condition has been called 'infection immunity'. It must be noted, however, that such immunity is only of a very limited nature, since the refractory state of the skin is not necessarily accompanied by absence of lesions in other parts of the body. Analogous phenomena are presented in tuberculosis. In other infections, especially those caused by protozoa, a similar state is met with, e.g. in malaria, where during latency of the disease attempts at superinfection fail. Again, in piroplasmiasis of cattle, after recovery from the acute attack the blood remains permanently infective for fresh animals, although the original host presents no further signs of disease. This state of non-sterilising immunity is sometimes called 'premunition'. Both in malaria and syphilis the presence of antibodies to the parasites can be demonstrated, it would appear, however, that in these conditions other factors also conduce to immunity, such as a heightened capacity of the tissues, especially the reticulo-endothelial system, to destroy the parasites, possibly accompanied by the development of some form of tolerance to those which persist. To this the term 'tissue immunity' is applied. Another example of tissue immunity is the increased bacteriostatic property exhibited towards tubercle bacilli by the mononuclear phagocytes of immunised animals in the absence of immune serum. This has been demonstrated experimentally by implanting the phagocytes loaded with bacilli into the anterior chamber of the eyes of normal rabbits (Lurie). The nature of such forms of immunity is obscure, however.

NATURAL IMMUNITY

The consideration of this subject has been placed after that of acquired immunity, as the latter supplies facts which indicate in what direction an explanation of the former may be looked for. Certain aspects of the subject have been discussed already in relation to resistance and susceptibility to infection. There may be said to be two main facts with regard to natural immunity. The first is, that there are very many species of bacteria—the so-called *non-pathogenic organisms*—which are practically incapable, unless

ing living bacteria. The second fact is, that there are certain bacteria which are very virulent to some species of animals, whilst they are almost harmless to others, the anthrax bacillus, the glanders bacillus, and the gonococcus may be taken as examples. Now it is manifest that natural immunity against such organisms might be due to a special power possessed by an animal of destroying the organisms when introduced into its tissues. It might also possibly be due to an insusceptibility to, or power of neutralising, the toxins of the organisms, for the study of the various diseases shows that toxins (in the widest sense of the term) are the agents by which morbid changes are produced, and that toxin-formation is a property common to practically all pathogenic bacteria. As a matter of fact, natural immunity in most cases is manifested against *infection*, i.e. consists in a power possessed by the animal of destroying the living bacteria when introduced into its tissues. Such a power may exist though the animal is still susceptible to the separated toxins. These two factors will now be dealt with separately.

1. Variations in Natural Bactericidal Powers. The fundamental fact here is that a given organism may be destroyed rapidly in one animal, whereas in another it may multiply quickly and produce morbid effects. The special powers of destroying organisms in natural immunity have been ascribed to (a) phagocytosis, (b) action of the serum by itself, and (c) combined action.

(a) *Phagocytosis*. The part played by the leucocytes and the phagocytes of the reticulo-endothelial system, which are accumulated especially in the spleen, liver, bone marrow, and lymph glands, in removing organisms from the blood and lymph, has already been dealt with (pp 58, 98). The chief

immunity present, but are probably in themselves capable of explanation. The fundamental observations of Wright and Douglas showed that an essential factor in phagocytosis is the labile opsonin of normal serum, which has combining affinities for a great many organisms, as already stated. It is possible, too, that in addition to acting on the organisms the serum may also contain 'stimulins' which enhance the activities of the phagocytes (A Wright). In many instances, however, natural antibodies are concerned in the opsonic action, as in the case of the protective effect of normal fowls' serum against pneumococci in mice (Bull and McKee). But the all-important fact is that whether phagocytosis occurs or not appears to depend upon

certain bodies in the serum. As yet, we cannot say whether the phagocytosis in a given serum, observed according to the opsonic technique, always runs parallel with phagocytosis in the tissues of the animal from which the serum has been taken, but it has been found in many instances that the results *in vivo* and *in vitro* closely correspond. In the non-occurrence of phagocytosis seen when an organism acquires increased virulence, two main factors are concerned. On the one hand, as has been demonstrated in certain instances, the insensitiveness to opsonic action may be associated with recognisable changes in the organism, notably the formation of a capsule. On the other hand, the condition of virulence is sometimes due to the production by the organism of substances such as aggressins which have an inhibitory effect on phagocytosis; in the case of capsulate organisms the capsular material diffusing out into the surrounding medium has this effect. Ingestion is, however, only the first stage in the process; intracellular destruction is the second, and is of equal importance. But the mechanism by which the phagocytes of the reticulo-endothelial system destroy ingested bacteria is still obscure. What may be called intracellular bactericidal action probably varies in the case of phagocytes of different animals, and, further, bacteria sometimes survive the cells which have ingested them, the latter undergoing necrosis and disintegration. In other instances, the organisms do not appear to suffer from their intracellular position; an example of this is afforded in the case of tubercle bacilli ingested by mononuclear phagocytes of normal animals. Variations in the susceptibility of organisms to the intracellular bactericidins is thus another important factor in natural immunity; but, so far, these differences cannot be explained.

(b) *Bactericidal action of the serum* Lister originally observed that putrefactive bacteria did not grow readily in blood serum. The bactericidal action of serum was specially studied by Nuttall, and later by Buchner and Hankin, who believed that this property was due to certain substances derived from the spleen, lymphatic glands, thymus, and other tissues rich in leucocytes. To these substances Buchner gave the name of *alexins*. Then the question naturally arose as to whether this bactericidal power varied in different animals in proportion to the natural immunity enjoyed by them. The earlier experiments of Behring appeared to give grounds for the belief that this was the case. He found, for example, that the serum of the white rat, which has a remarkable immunity to anthrax, had greater bactericidal powers than that of other animals examined. Further investigation, however, has shown that this is not an example of a general law; and that the bactericidal action of the serum does not vary *pari passu* with the degree of immunity. In some cases non-pathogenic and also attenuated pathogenic bacteria can be seen to undergo rapid solution and disappear when placed in a drop of normal serum. Normal human serum is only slightly bactericidal towards the gonococcus, while this organism is readily killed by the sera of many other mammals which are insusceptible to the infection (Abdoosh). Again Schütze and others have shown that strains of mice which are markedly resistant to salmonella infection, possess more natural antibody to these organisms than do susceptible strains. However, in the case of many pathogenic organisms, such as staphylococci and streptococci, the serum may have no direct bactericidal effect at all.

As regards the mechanism of the bactericidal action of normal serum, it has been explained earlier that Buchner's alexins correspond with Metchnikoff's cytases and Ehrlich's complements, usually acting in association with a natural analogue of immune-body. As already stated, complement does not increase in amount during immunisation; also there is no satisfactory

evidence that its source is the leucocytes or that it is set free from them only after the blood is shed, as has been maintained by various French writers. In the case of the hæmolytic action of a normal serum, it has been shown that, in addition to complement, as a rule a natural immune-body is also concerned, the process being similar to that in the case of an artificially developed hæmolytic serum. In many instances an analogous condition obtains also in the case of normal bactericidal sera. For example, ox's serum, heated at 55° C, contains a natural immune-body to *V. cholerae* which can be activated by the addition of complement so as to produce a bactericidal action, though complement is by itself without any such effect. Mackie and Finkelstein have shown that a normal serum may contain a number of such natural immune-bodies which are specific in character, and there may even be differences with respect to strains of the same species of organisms. A difficulty emphasised by Gordon *et al* is the number of such natural antibodies required in order to account for all the bactericidal, etc., effects produced. Accordingly, it may be supposed also that which are relatively non-specific.

Variations in bactericidal power of the serum due to complement as tested *in vitro*, however, do not explain the presence or absence of natural immunity against a living organism, e.g. the anthrax bacillus. In some cases, for example, it has been found to be considerable, while the organisms flourish in the body and the animal has no immunity.

In addition, bactericidal action has been shown to be possessed by certain substances extracted by physical or chemical means from leucocytes—*leukins*, and also from platelets—*plakins*. (It has been shown that the blood-platelets very rapidly disintegrate when exposed to certain conditions.) When injected into certain cases (

complement, not invariably destroyed at 55° to 60° C. for half an hour, and are little affected by salt concentration. Moreover, their action is a direct one, immune-body not being concerned, and is efficient on different organisms from those killed by complement. Their activity, however, is found to vary greatly in the case of different animals. It is generally held that bodies of the leukin class are set free only on destruction of the cells, though some writers consider that this may occur also by a process of secretion. But within the cells such substances apparently play an important part in the intracellular killing of organisms, and variations in resistance may sometimes depend on them. Another type of bactericidal substance exists normally in serum and acts especially on *B. anthracis* and certain other Gram-positive organisms, which Pettersson has described as β -*lysin*. This differs from complement in many respects, being especially more thermostable, it consists of several components. Thermostable bactericidal substances for organisms of the coli, salmonella, typhoid, and dysentery groups have also been found in serum. *Lysozyme* occurs in various secretions, especially tears, and in tissues.

the combined action of serum and phagocytosis on bacteria. In this way the summation effects of extra- and intra-cellular antibacterial mechanisms are obtained. This may be done by exposing the organisms to the action of whole blood. By this method a definite correlation has been found to exist between the bactericidal power of the blood of rats for *B. pestis* and immunity to

infection with that organism (Malone, Avari and Naidu). Similarly, Bull and Tao have shown by using citrated whole blood inoculated with varying doses of pneumococci, that in the case of an insusceptible species (chicken) the inoculum required to yield growth of the organisms is over one million times greater than is required with the blood of a susceptible species (rabbit). Instead of whole blood, mixtures of serum and leucocytes may be allowed to act on the organisms. The enhanced bactericidal effect of such mixtures as compared with that of the separate constituents is probably due to various factors (Mackie *et al.*).

2. Variations in Natural Susceptibility to Toxins. Here one starts with the fundamental fact, incapable of explanation, that toxicity is a relative thing, or, in other words, that different animals have different degrees of resistance or non-susceptibility to toxic bodies. In every case a certain dose must be reached before effects can be observed, and up to that point the animal has resistance. This natural resistance is found to present very remarkable degrees of variation in different animals. The great resistance of the common fowl to the toxin of the tetanus bacillus may be mentioned, and large amounts of this poison can be injected into the scorpion without producing any obvious effects. The high resistance of the rat to diphtheria toxin is another striking example. This variation in resistance to toxins applies also to those which produce local effects, as well as to those which cause symptoms of general poisoning. Instances of this are furnished, for example, by the vegetable poisons, ricin and abrin, by the snake poisons, and by bacterial toxins such as that of diphtheria. The insusceptibility of the new-born to certain toxins has been referred to (p. 51). According to Ehrlich's view it may be due to the want

of the toxophore group of the toxin; or, on the other hand, supposing this affinity to exist, it might be due to an innate non-susceptibility to the action of the toxophore group. Investigations have been made in order to determine the combining affinity of the nervous system of the fowl for tetanus toxin, as compared with that obtaining in a susceptible animal, but the results have been somewhat contradictory. Accordingly, a general statement on this point cannot at present be made, though in all probability variations in the susceptibility to the toxophore group will be found to play a very important part. It was shown by Muir and Browning by means of hæmolytic tests that the toxic activity of complement, after it has been fixed to the corpuscles, varies very much, in some instances an amount of complement which would rapidly produce complete lysis of one kind of corpuscle, may have practically no effect on another, even though it enters into combination. These results are of importance in demonstrating how the corresponding cells of different animals may vary in sensitiveness to toxic action.

Origin of Natural Antibodies and Immunity. In certain diseases, e.g. diphtheria and scarlet fever in this country, susceptibility is greatest in childhood and diminishes with age. The increase in resistance is due chiefly to the appearance in the serum of antitoxins for the toxins of the causal organisms, although the individuals have never suffered from clinically recognisable attacks. According to one view the explanation of the occurrence of these antibodies in the serum is that the corresponding organisms are widely prevalent in the community and therefore in the course of time most persons acquire a series of 'subclinical' infections which lead to their development. Observations on the behaviour of diphtheria in a semi-isolated community strongly support this view (Dudley). On the other hand, from a study of the distribution of natural lysins and agglutinins for sheep and

rabbit red cells respectively in the blood of children at different ages, Friedberger has argued that the presence of antibodies is associated with maturation of the tissues. This explanation may not be conclusive, since those animals are so widely consumed as food, but the natural lysin for sheep's corpuscles in rabbit's serum behaves similarly. Gibson has made a like observation in regard to natural agglutinins, where infection with the organisms could be excluded practically. Also, certain antibodies are formed under circumstances which exclude a reaction to a specific antigen. Such are the hæmolytic immune-body for ox's red corpuscles in normal guinea-pig's serum; also the agglutinin for *B. mallei* in the serum of horses in Great Britain although glanders does not exist there (Lovell). Further, the different anatomical distribution of the same infection at varying ages, e.g. tuberculosis, and the predilection of certain infections for particular age groups, indicate clearly that maturity of the tissues plays an important part in determining the incidence of infections. Again, the tissues may naturally possess defensive mechanisms which are not explicable on the analogy of an acquired immunity. For instance, Flexner found that certain monkeys were refractory to inoculation with poliomyelitis virus placed on the nasal mucous membrane, but these animals possessed no special resistance to intracerebral inoculation, and their serum had no action in neutralising the infectivity of the virus.

If we review the whole subject of natural immunity it appears that variations in such immunity shown by different species depend in the main upon

natural immunity depend on variations in the susceptibility of the tissues to toxins (in the widest sense) formed by the bacteria.

MASS OR HERD IMMUNITY : EXPERIMENTAL EPIDEMIOLOGY

Hitherto the reactions of the individual have been taken into account

diseases among masses was almost purely descriptive. Certain of these diseases were known to become epidemic at intervals, the numbers of cases at such times being large as compared with that in the intervals. A disease with very regular periodic epidemics is measles in this country; epidemics of influenza and poliomyelitis, on the other hand, are highly irregular in their occurrence. From the observations of Farr and others, it appears that epidemics generally follow a regular course as regards their onset, acme, and decline, also the subsidence of an epidemic occurs long before the number of susceptibles is exhausted. It seems likely, therefore, that the onset and progress of an epidemic are due to some initial, almost explosive increase in infectivity or pathogenicity of the causal agent, which later diminishes again (Brownlee). Of course, alterations in the susceptibility of the population might also be responsible, but it is difficult to conceive that a change of this nature on the part of the hosts can be world-wide, as would have to be the case in order to explain a pandemic like that of influenza in 1918. So far, however, it has not been possible to investigate the causal agent of a natural epidemic from this point of view, although cyclical changes in virulence of the virus of poliomyelitis under conditions of experimental transmission have

been established. On the other hand, the regular recurrence of epidemics of measles at about two-yearly intervals may well depend on the accumulation of highly susceptible children born in the intervals between epidemics. In the case of endemic infections such as tuberculosis, the alterations in prevalence and severity take place over much longer periods. The circumstances of a natural population are so complex as to render a detailed analysis of the conditions almost impossible. Factors affecting the hosts, such as emigration and immigration, varying susceptibility due to sex and age, occupation, nutrition, etc., must all be considered; also accidents leading to excessive exposure must be regarded, *e.g.* where a defective drain from an isolation hospital has permitted sudden access of infective matter from a case of enteric to the water supply. All these points obviously influence the spread of infections. It must also be borne in mind that the practice of isolation has had little effect on the spread of epidemic diseases, chiefly, no doubt, on account of the fact that cases are clinically recognisable only at certain stages and also that a proportion cannot be recognised at any time in their course.

In order to study the problems under somewhat simpler conditions the behaviour of infective diseases in semi-closed communities has been investigated, especially in the case of diphtheria, by Dudley; also epidemics have been set going in collections of small animals such as mice, and the influence of adding fresh susceptible individuals at different rates or of adding immunised animals has been studied by Greenwood and Topley in this country and by Webster *et al* at the Rockefeller Institute in New York. The procedure adopted was to infect adult mice by feeding, *e.g.* with mouse typhoid or *pasteurella* bacilli, and then to introduce similar uninfected animals into the infected community, the rate and number of introductions being varied in different experiments. When normal mice were introduced it was found that practically all became infected eventually and that soon deaths among these occurred, which after a time gradually diminished in number until a point was reached at which the survivors had a somewhat better expectation of life than more recent immigrants. The weeding out of susceptibles probably contributes in part to this resistance, but it appears to be due chiefly to a process of immunisation, especially since it tends to be specific for the organism concerned. In the case of ectromelia, a virus infection in mice, a fairly high degree of immunity develops eventually. However, waves of exceptionally high mortality occur during which many old survivors die, *i.e.* epidemics supervene in a population in which the disease is fairly static.

The next question is the influence produced upon the fatality by adding immunised animals to the infected herd. With mouse typhoid vaccines a distinct but not high degree of protection was conferred in the sense that the treated animals survived introduction into the infected community longer than did normal ones. But a large proportion became infected nevertheless. Thus, once a herd has been infected by mouse typhoid, the introduction only of immunised animals does not lead to disappearance of the infection. The introduction of bacteriophage has also been ineffective in this respect. The protection conferred by a specific vaccine was much greater in the case of ectromelia, at least in the early periods after entry to the herd.

The experiments did not throw light on the hypothesis that natural epidemics start from the development of an 'epidemic' strain of the organism, although variations in infectivity were found to occur in the organisms isolated during the course of experimental epidemics. But they serve to demonstrate the distinction between the capacity of a strain to spread naturally from host to host and its capacity to cause severe illness in infected individuals. It was found that an organism might infect a large proportion

of animals exposed to it and nevertheless cause only small fatality. Accordingly it appears likely that a strain which is capable of causing an epidemic must combine both the properties of infectivity and pathogenicity.

SUPERSENSITIVENESS, ANAPHYLAXIS, AND ALLERGY

The term *supersensitiveness* is applied to states where, as compared with the normal, there is increased susceptibility or reactivity to substances introduced into the body, either parenterally or through the alimentary canal. The condition may be manifested by either local or general disturbances, and

minute amount of foreign serum may give rise to such effects. A particular individual, however, is likely to be supersensitive to only one of these various substances. Such supersensitiveness has been termed *natural*, but it is doubtful whether in most cases it does not result from previous contact with the substance, accordingly, it may come into the same category as the next examples. The increased reactivity of patients or animals suffering from an infection to the products of the infecting organism is a noteworthy example of *acquired* supersensitiveness. Thus we have the tuberculin and mallein reactions, and here both local and general effects are met with. Jenner probably described the first instance of allergy in infective disease when he noted that in an individual who had suffered from cowpox and therefore was immune to smallpox, inoculation of the skin with variolous matter led to an inflammatory reaction at the site more rapidly than did a similar inoculation in a susceptible person.

It has come to be recognised that there is a special form of supersensitiveness which is induced by the injection of substances with antigenic properties, the toxic phenomena becoming manifest only on a further injection of the same antigen after a suitable interval of time. In this form the disturbances are now known to be the result of the interaction of antibody and antigen. Thus, while the phenomena in their characters and results present a striking contrast to the state of immunity, they are in their mode of production closely related to the latter condition. To this form of supersensitiveness the term 'anaphylaxis' is generally applied, and it is advisable to restrict its use in this way. The substances which have been found to have the property of calling forth the anaphylactic state—anaphylactogens—are of various kinds, including bacteria and their toxins, animal poisons, and a great many foreign proteins, e.g. those of serum, milk, egg albumin, plant products, peptone (as a constituent of bacteriological media), etc. They are chiefly of protein nature, and they are probably coextensive with the substances which act as antigens in immunity reactions. It is important to note that many of the substances which act as anaphylactogens are on their first injection perfectly harmless. In order to produce anaphylactic shock in sensitised animals it is not always essential to inject the complete antigen, since the corresponding haptens may be effective. Thus, in animals sensitised with antisera to *B. lactis aerogenes* the injection of the specific carbohydrate substance produced anaphylactic shock (Tomesik and Kurotchkin). Similarly Landsteiner *et al.* by injecting animals with a compound of protein with an azo-dye, rendered them supersensitive to the dye by itself. It was shown also that supersensitiveness (both local and general) can be produced by simple chemical substances, e.g. salvarsan, and anaphylactic shock may follow intravenous injection of this drug in a supersensitive person. Presumably the drug

combines with some protein in the tissues, and this complex acts as the anaphylactogen

Many of the examples of supersensitiveness, e.g. the tuberculin and allied reactions, have not yet been proved to depend on the interaction of antigen and antibody.

To such supersensitive conditions of hay fever, asthma, susceptibility to certain articles of diet, etc., he has given the name of 'atopy' (strange disease). The group is, however, not a homogeneous one, and in most instances the mode of production is obscure; the nature of each must be investigated separately. The toxic effect produced by injecting an antiserum to an animal's own serum proteins is a phenomenon related to anaphylaxis. A certain amount of evidence has been brought forward that puernerale eclampsia is produced by the same mechanism. It is not yet clear whether these have the property of establishing a permanent immunity. Knowledge of such problems is still very defective.

Anaphylaxis. At a comparatively early date it was shown by the fact that the sum of the doses might amount to only a fraction of a lethal dose. Richet investigated a similar phenomenon in the case of a toxic substance obtained from the tentacles of actiniae, to which, from its action, he gave the name of 'congestin'. He found that a certain time-interval between the injections was necessary; that after the second injection the symptoms occurred with remarkable rapidity, and that they had passed.

He also found that the condition lasted several weeks at least. Arthus observed that after repeated injections of horse serum in rabbits, a stage was reached at which an additional subcutaneous injection produced marked œdema and even necrosis, while an intravenous injection, harmless to an untreated animal, brought about a fatal result. The period of active research on the subject, however, may be said to date from the discovery of what is now known as the 'phenomenon of Theobald Smith'. This observer found that guinea-pigs which had been treated with a neutral mixture of diphtheria toxin and antitoxin might, after a certain interval of time, succumb on being injected with a quantity of normal horse serum. It was afterwards shown—especially by the researches of Otto and of Rosenau and Anderson—that the sensitising agent had really nothing to do with the toxin or antitoxin, but was due to the constituents normally present in the serum. In the study of anaphylaxis various sera and proteins from other sources were originally employed. The guinea-pig is the most suitable test animal for the study of anaphylaxis. The dog is also used, but the relative susceptibility of the latter is less than a hundredth of that of the guinea-pig. In the case of mice or rats, it is difficult to bring about severe anaphylactic effects. Anaphylaxis has the character of specificity, apparently within corresponding limits to immunity (p. 79)—that is, it is manifested only on the reinjection of the same protein substance as that used in the first instance. It is to be noted that the minimum amount of serum necessary to bring about the symptoms of fatal anaphylactic shock is much greater (it may be one thousand times) than the original sensitising dose. It was probably owing to neglect of

this fact that Besredka concluded that the sensitising and the toxic factors were different when he found that serum heated to a certain temperature still had the power of inducing the condition of anaphylaxis, but was no longer capable of bringing about the toxic phenomena when injected into an anaphylactic animal. The effect of the heating was to reduce the activity of the serum to such an extent that a dose sufficient to produce shock was not administered, whereas enough was given to bring about sensitisation.

Phenomena of active anaphylaxis in the guinea-pig There is, first of all, the sensitising injection, a minute amount, e.g. 0.01 c.c. of foreign serum, injected subcutaneously, though even 0.000001 c.c. has been found sufficient in certain instances, other methods of parenteral injection may also be employed. After a certain number of days, usually ten as a minimum, anaphylaxis has been established, and the test for this is usually made by injecting by the intravenous route 0.5 c.c. of the same serum or even less, or intraperitoneally 5 c.c.—the *assaulting* dose. The former method brings about the result more rapidly. Subcutaneous injection is less effective and less certain. In the anaphylactic animal, especially after an intravenous injection of serum, acute shock occurs, which causes death usually within a few minutes. Restlessness, often accompanied by scratching of the nose and sneezing, is followed by evidence of collapse, urine and faeces are passed, the respiration is embarrassed; the animal falls over and, after a few convulsive movements, is dead. In the more protracted form of shock following an intraperitoneal or subcutaneous injection the animal becomes comatose and its temperature falls markedly and also the blood pressure. While anaphylaxis is not fully established till about the tenth day, it occurs gradually—not by crisis—as can be shown by disturbance of the temperature on reinjection of serum at a much earlier period.

Passive anaphylaxis There is also a passive anaphylaxis, as is shown by the fact that if a certain amount of the serum of an anaphylactic guinea-pig (or of one which contains antibody, even if it is not anaphylactic at the time) be injected into a normal guinea-pig, the latter becomes anaphylactic, so that the characteristic symptoms appear in it when the test amount of antigen (anaphylactogen) is injected. In the guinea-pig shock is produced with greatest certainty if an interval of some hours has elapsed between the injections; if the two injections are made at the same time there is frequently no result, but this is not invariably the case (H. R. Dean *et al.*). The occurrence of this interval is of importance in connection with the nature and mode of causation of anaphylaxis. In the rabbit, however, the symptoms appear almost at once if the two injections are given practically at the same time. The antiserum need not be derived from the same species as the animal which is to be rendered passively anaphylactic; thus the guinea-pig is passively sensitised by antiserum from the rabbit or man, but often not by one from the horse (Avery and Tillett). Passive anaphylaxis usually disappears after a few weeks at longest, whereas active anaphylaxis has been observed after more than two years, here also there is an analogy between anaphylaxis and immunity. Another interesting observation has been made, namely, that the yot and the condition m.

Reversed passive produced, e.g. in rabbits, by injecting an antigen, horse serum, intraperitoneally and after an interval of about four hours following this by an intravenous injection of the corresponding antiserum, derived from the rabbit (Opie and Furth). Kellet has shown also that anaphylactic shock may be produced by injecting guinea-pigs with foreign serum (antigen) followed after

an interval by a large dose of the corresponding antiserum from the rabbit, both injections being given intravenously. According to Zinsser and Enders, however, only exceptional animals behave in this way and there need be practically no interval between the two injections.

Anti-anaphylaxis and desensitisation. It is possible to produce a condition of anti-anaphylaxis. If, for example, the small sensitising dose of horse serum is injected into a guinea-pig, and before anaphylaxis is established (*i.e.* some time before the tenth day) another injection consisting of a considerable quantity of horse serum is given, the animal is then found to be non-susceptible (refractory) to further injections for a considerable period of time. During this period its serum contains antibody (precipitin) to the injected serum and is capable of conferring passive anaphylaxis. Eventually, however, the state of anaphylaxis becomes established, although it can be deferred indefinitely by repeated injections of the serum. Also, non-susceptibility to a further dose of serum supervenes for a time when an animal recovers from anaphylactic shock. Further, if anaphylaxis exists, the serious effects may be avoided by injecting a small dose of serum, insufficient in itself to bring about typical symptoms, and following this by the injection of gradually increasing doses. In this instance desensitisation is said to have been brought about (*vide infra*); according to Morris, the factors concerned here may be largely non-specific, since saturation of antibodies is not chiefly responsible. Insensitiveness may also be induced by ether anaesthesia at the time the assaulting dose is given, and by drugs such as adrenaline or atropine, or by the injection of hypertonic saline, or of serum of a species other than that with which the animal has been sensitised, all given shortly beforehand.

Symptoms and pathological changes in anaphylaxis. With regard to the symptoms and pathological changes in anaphylaxis, two facts are of importance. The first is that in a given species of animal these are of the same nature, no matter by what antigen the condition has been produced. The second is that the anaphylactic phenomena taken as a whole vary according to the species of animal. This does not mean that the condition varies essentially in nature, it probably indicates merely that different tissues are more susceptible in different animals. The symptoms of acute anaphylaxis in the guinea-pig have already been described, and the chief change found *post mortem* is an acute over-distension of the lungs which is due, as was first shown by Auer and Lewis, to a spasm of the muscle fibres in the fine bronchi and alveolar passages. In the *rabbit* the acute symptoms are somewhat different, there being an absence of marked dyspnoea. Convulsions may occur and the heart usually continues to beat after breathing has stopped; or coma may develop. The arterial blood pressure falls greatly. The chief post-mortem change is a great accumulation of blood in the splanchnic venous system, this results from contraction of the pulmonary arterioles. In the *dog* there are symptoms in connection with the alimentary canal, vomiting and evacuation of the bowels and bladder, followed by muscular weakness, a shock-like fall of the blood pressure and coma. *Post mortem*, the chief changes are found to be a great enlargement of the liver due to accumulation of blood and an intense engorgement of the portal area. In both the rabbit and the dog there is marked leucopenia, and this is attended by diminution in the coagulability of the blood. The presence of subserous capillary hæmorrhages is another feature. In the *man*, anaphylaxis is less acute, it requires for its production a longer period of sensitisation leads to a 'protracted' or less acute anaphylaxis. On acute fatal anaphylaxis in *man*, comparatively few authentic cases have been

recorded (Waldbott); but in one case carefully studied and described by H. R. Dean where death occurred in little more than one hour, the main symptoms and pathological changes corresponded with those observed in the dog.

Mechanism of anaphylaxis. While it is now admitted by all that anaphylaxis depends on an interaction of antigen and antibody (anaphylactogen and anaphylactin), there has been much diversity of opinion as to the site and mode of its occurrence. Two chief views have been put forward. According to one, the combination takes place in the tissues and there the disturbance is produced; according to the other, the poison is formed by the combination in the circulating blood and thus affects various parts of the body—the *cellular* and *humoral* theories respectively.

It is definitely established that in an anaphylactic animal the antibody (anaphylactin) is present in the tissues, and toxic effects result when antigen is brought into relation with it. Schultz showed that isolated segments of the intestine of a sensitised animal responded to the application of the antigen in a specific way by contraction, and this result was confirmed, and amplified in extensive experiments by Weil and by Dale. Using the muscle of the uterus of the guinea-pig from which the blood had been removed by perfusion, Dale showed that when the specific antigen is brought into contact with it the muscle undergoes sudden contraction, which begins to pass off after a few minutes, thereafter the muscle is desensitised and does not respond further. He compared the effect to that of a powerful stimulant drug, and considered that it did not resemble enzyme action or any form of proteolysis, etc. It has been shown by various workers that the antiserum in anaphylaxis corresponds closely in many respects with precipitin. In fact, some hold that the two substances are identical, since for the production of passive anaphylaxis it is not essential that the serum should be derived from an animal in the anaphylactic state, provided that it contains a precipitin for the test antigen. The view as to the reaction taking place in the tissues is in harmony with other results. The fact that in an actively sensitised guinea-pig the symptoms appear at once on injection of the antigen, whereas in passive anaphylaxis a considerable time must usually elapse after the antiserum is injected before the animal develops maximal sensitiveness to the antigen, is a stumbling-block to the theory that a poison is formed by the interaction of the two substances in the blood. It seems, however, capable of explanation on the view that the interval of time is necessary for the antibody in the serum to be fixed in the tissues. The so-called 'replacement' experiments, in which the blood of the sensitised animal is replaced by normal blood, lead to a similar conclusion as to the site of the reaction, in such experiments the animal is still anaphylactic on injection of antigen, and the same is true of its separated tissues. Attempts to show that the blood of an animal in acute anaphylactic shock is toxic to another animal have given negative results. But blood obtained directly from the liver of dogs in a state of anaphylactic shock produces anaphylactic effects when introduced into the circulation of a normal dog. This is in accordance with the view that in these animals the liver is the chief seat of disturbance, since it has been found that in a sensitised dog in which the liver vessels are clamped shock does not occur on the injection of the foreign protein, but after releasing the clamps the usual effects develop (Manwaring).

Dale suggested that the phenomena of anaphylactic shock might be due to some disturbance of the colloid state of the muscles. He then brought forward evidence which rendered it likely that the anaphylactic reactions are due, in part at least, to the effect of histamine liberated as a result of cell

Experiment :

Rosen, Reidberg and Nagel in

The anaphylactic reaction was --
sensitised guinea-pig
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in the

also been found that the vascular reaction in anaphylactic dogs is accounted for by the content of the blood in histamine (Dragstedt *et al.*). In support of the part played by histamine is the finding that drugs which antagonise it prevent anaphylactic shock. It appears likely, however, that other substances liberated in the body contribute to the anaphylactic reaction, thus Kellaway and Trethewie have shown that a 'slow-reacting smooth muscle-stimulating substance' appears in the fluid obtained by perfusing sensitised lungs with antigen. It differs from histamine in its behaviour, but resembles a substance formed from tissues by enzymes of snake venoms. The diminution in the coagulability of the blood, which is a noteworthy change, *e.g.* in the dog, results from the setting free of heparin from the liver (Jaques and Waters). The marked leucopenia of the peripheral blood, which is in general a striking feature, is due to the accumulation of the leucocytes in the lung and other organs, in a similar manner to what is seen after the injection of a dose of peptone or other substances into the blood.

The humoral theory, that anaphylaxis is due to a toxin formed in the circulating blood by antigen and antibody, was originally put forward by Richet, who called the toxic product 'apotoxin'. Friedberger explained the phenomena as resulting from the reaction of antigen and antibody.

protein introduced
He showed that
plus precipitin)

On being separated from the precipitate and injected into an animal, causes symptoms like those of anaphylaxis; this body he called *anaphylatoxin*. He showed further that anaphylatoxin is produced by the action of complement on bacteria treated with their anti-serum, and also by the action of normal serum alone on bacteria, and even on coagulated serum. The possibility of proteolytic action by complement has, however, been called in question, and corresponding symptoms have been produced in other ways. For example, complement-containing serum on contact with kaolin, agar, etc., has been found to acquire similar properties, and it is generally held now that the various anaphylatoxins are not specific in their action. Dale and Kellaway showed that the effects produced by the anaphylatoxins differ from those in true anaphylactic shock, evidence of injury to the vascular endothelium and lysis of platelets being more conspicuous in the action of all of them. Further, they found that tolerance to anaphylatoxin can be acquired and that this does not involve desensitisation of the anaphylactic animal, on the other hand, desensitisation does not make an anaphylactic animal non-susceptible to the action of anaphylatoxin. The above is a very complicated, and while the tissues leads to the essential immunity that toxic bodies formed in the blood itself may play a part cannot be excluded. Rabbits which receive a suitable intravenous dose of antiserum followed at once by the corresponding antigen develop

anaphylactic shock, which suggests that the reaction between the two substances occurs in the blood. This is in agreement with the finding that rabbit's leucocytes contain a relatively large amount of histamine, which presumably is discharged into the plasma as the result of damage to these cells and so causes the anaphylactic shock. The fact that rabbits which have been repeatedly injected with antigen until their blood contains large amounts of antibody are nevertheless sensitized, is also in favour of the blood rather than the tissues being the site of the reaction. In anaphylaxis there is often a marked fall in to its fixation in the usual way by

Allergy. I

it the phenomena of anaphylaxis just described are of the same nature as the supersensitiveness or allergy manifested by infected subjects to the products of the infecting organism, *e.g.* that to tuberculin, mallein, brucellin, trichophytin, etc (often referred to as 'allergy of infection'); though in all probability they are at least similar in essence. It has been held as a distinction that this supersensitiveness in infections to bacterial products could not be transferred to another animal; and there is no conclusive evidence that this is possible in the case of tuberculin. Other features of this type of reaction are that the effects develop slowly (in contrast to the prompt appearance of both local and general effects in anaphylaxis) and the smooth muscle of the allergic animal is not thrown into sudden contraction on contact with the sensitising agent. According to one view, which appears to have most in support of it, the phenomena of supersensitiveness of tuberculous patients to tuberculin is due to the combination of the injected antigen with molecules of antibody resident in certain tissue cells, the so-called 'sessile receptors'. In favour of this view is the observation of Rich and Lewis that tissue cultures *in vitro*, which have been derived from animals supersensitive to tuberculin retain this supersensitiveness after repeated subculturing. Friedberger, however, held that the facts can be equally well explained by the combination, which occurs either locally or generally, of the antigen with antibody in the serum, this combination when acted upon by complement giving rise to the poisonous substance. Others, again, consider that there is not sufficient evidence that an antibody is concerned, and that local allergy is of a different nature. According to Dienes, specific supersensitiveness of the tissues is the first manifestation of reaction and is an indicator of immunisation. But Rich and others have demonstrated that in animals which are both immune and supersensitive, desensitisation may be effected without removing the immunity, also, immunity may exist without supersensitiveness, as in the case of rabbits passively immunised to the pneumococcus and inoculated intracutaneously. Again, it would appear that certain antigens are more prone to develop supersensitiveness than immunity and vice versa. At present it is impossible to state definitely whether or not supersensitiveness may contribute to immunity in certain conditions. Further details are given in connection with the special chapters. There is no doubt, however, that the supersensitive state must play an important part in the clinical manifestations of many diseases. For example, the sensitiveness of tuberculous patients to tuberculin indicates that the symptoms and lesions in this disease are produced by the absorption from the tuberculous foci of a smaller amount of toxin than would be necessary to produce corresponding effects in a normal individual. Also, the sensitiveness of the conjunctiva in typhoid fever to the products of the bacillus suggests that in this disease, too, supersensitiveness plays an important part. It appears at least likely that bacterial products set free in the body in infections gradually produce a state of supersensitiveness which

is closely similar to a true anaphylactic state. The term allergen is applied generally to agents which excite allergic reactions ; these include also vegetable and animal products in dusts, chemical compounds, and physical agencies, such as heat, cold, and sunlight. The effects may follow contact with any body-surface as well as introduction into the tissues. As a rule, in the human subject, allergy is confined to a particular substance, and the clinical manifestations (' shock organ ') depend on the individual.

The phenomena of hay fever probably belong to the class of allergic disorders, being apparently the result of repeated absorption of vegetable proteins ; a similar statement may apply to the sensitiveness to articles of diet which is manifested by disturbances of the alimentary tract, urticaria, etc. Asthma appears to be the reaction in certain allergic individuals to substances absorbed by the respiratory or alimentary tract. In all such cases, however, the possibility of a natural supersensitiveness as an *idiosyncrasy* must be considered. Supersensitiveness of infants to cow's milk has been recorded, and the history of cases indicates that such is due to the absorption from the alimentary tract of protein molecules, or, at least, of derivatives which may act as antigens. The possibility of such an occurrence is shown by the work of Ehrlich on the vegetable toxins, ricin and abrin ; and Walzer has demonstrated by sensitisation methods the presence in the serum of incompletely digested proteins after their ingestion by normal adults.

Atopy In a number of these conditions of idiosyncrasy in which supersensitiveness to certain substances appears clinically as hay fever, asthma, dermatitis (eczema), or susceptibility to certain articles of diet, it has been shown that the serum contains a ' reagin ', which when injected into the skin of a ' receptive ' normal person may produce local sensitiveness, so that on introducing the substance later (twenty-four to forty-eight hours) at the same site (or into the blood) a local inflammatory reaction develops (Prausnitz-Kustner reaction). Attempts to demonstrate neutralising properties in the reagin, or other characters of antibodies, have failed, however. On the other hand, following injections of pollen into patients supersensitive to this material an antibody has been shown to develop in the serum, which ' blocks ' the reagin. Atopy appears to be restricted to man ; and a marked hereditary predisposition exists. It is doubtful if atopy constitutes a class by itself.

Shwartzman phenomenon. A striking phenomenon described by Shwartzman, is related to allergy. The intradermal injection of bacteria or their products in rabbits causes local changes such that if after an interval, e.g. of twenty-four hours, an intravenous injection of filtrates from cultures of the same or other organisms be given, an acute reaction is provoked in the treated area of skin, this is characterised by hæmorrhage and necrosis. The effect may also be provoked by starch given intravenously (Freund), or when the animal has previously been rendered supersensitive to some antigen, an intravenous injection of the latter may provoke the reaction at the prepared site. Accordingly, a mechanism is suggested which may account for the development of focal lesions owing to some intercurrent infection, although the explanation is obscure.

The Serum Disease in Man. Apart from acute anaphylaxis in man referred to above, symptoms of milder degree are not infrequently observed after the injection of foreign serum for therapeutic purposes ; they were first fully described as a syndrome by Pirquet and Schick. There is here also a period of incubation, of six to twelve days on the average, but it may be three weeks, after which, in a proportion of cases, a group of characteristic symptoms appears. The proportion affected depends on the dose of serum ; when amounts of 100 to 1,000 c.c. of unconcentrated serum are given nearly

all persons develop serum disease ; after a dose of under 10 c.c. about 10 per cent. are affected. The use of 'concentrated' antisera has reduced the incidence of serum disease, since smaller doses of protein are injected, and 'refined' sera are still less liable to produce such effects, as the antigenic power of the proteins appears to be diminished. There may be prodromal symptoms of serum disease, swelling and tenderness at the site of injection, and in the corresponding lymphatic glands, and the spleen may be enlarged. Thereafter general exanthemata appear ; these are usually of an urticarial type, but may be erythematous or morbilliform. There is usually moderate pyrexia of a remittent type, and sometimes œdema and slight albuminuria are present ; occasionally there are pains in the joints ; there is also in children often leucopenia, due to a fall in the number of polymorphonuclear leucocytes. These symptoms last for a few days and then disappear. Sometimes after an interval, which may extend to two weeks, there is a recurrence. Rarely, nervous complications (neuritis and muscular paralysis not related to the site of injection, or cerebral symptoms) have been described (Thompson *et al.*). Such are the phenomena of the serum disease after a single injection of the foreign serum. It is noteworthy that similar phenomena following a single injection of foreign serum rarely occur in animals.

There are, however, two other types of reaction described by Pirquet and Schick, namely, the immediate and the accelerated reactions. The *immediate* reaction is seen when a large dose of serum has been administered, and then after a certain interval of time another dose of serum is injected. This interval is usually from twelve days to eight weeks, though sometimes as long as six months. The symptoms of the immediate reaction, which appear shortly after a subcutaneous or intramuscular injection, or at least within twenty-four hours, are an intense œdema locally, general exanthemata and pyrexia, though the general phenomena are often little marked. The symptoms pass off comparatively quickly, usually within twenty-four hours. Death has followed a small dose of serum, but this is very rare. When the injection has been given intravenously, the condition is more severe. The chief symptoms are shock, collapse, and dyspnoea, there is pallor or cyanosis, fall in the blood pressure, with feeble pulse, asthmatic symptoms, with cough and sometimes vomiting and severe headache—all setting in within a few minutes to two hours. The *accelerated* reaction is also seen after a second injection, and it may occur from six weeks up to many months after the first injection. In the case of the accelerated reaction there is an incubation period, but it is shorter than in the case of the first injection, being usually from five to seven days, the symptoms resemble those in the ordinary reaction as described above, but are of rather more acute onset and last a shorter time. In the interval from about the sixth week to the sixth month, there may occur both the immediate reaction and also, a few days later an accelerated reaction. The administration of a further dose of serum during an attack of serum sickness seems to be specially liable to cause serious effects. The occurrence of serum disease following the injection of fresh human serum from other individuals has been described (Dooley).

The phenomena of the serum disease in all probability depend upon the development of a reaction-body or antibody, as above described, though this view has been called in question by some writers. (Recurrent reactions would be explained by the successive development of antibodies to different antigenic proteins in the serum.) Cases are recorded where symptoms have occurred almost at once following the first injection, or after a time too short to correspond to a true incubation period. These, however, may be examples of 'natural' supersensitiveness, they have occurred especially in persons

with a history of supersensitiveness to contact with horses. But the facts described above, especially with regard to the phenomena after a second injection, point strongly to an antibody being concerned in the reaction. (The intracutaneous injection of a small quantity of the serum has been used as a test for supersensitiveness—*vide infra*). It is doubtful whether the 'thermal reaction', which sometimes sets in with rigor within half an hour after an intravenous injection of serum, belongs to the same class of phenomena.

Very serious local effects of the nature of the Arthus phenomenon (p. 128) have also been recorded rarely as the results of repeated injections of foreign serum into the tissues (Tumpeer; Ross).

Desensitisation. In view of the common use of curative serum, anaphylaxis has come to have considerable practical importance, especially in connection with intravenous injection, as by this route the dangerous dose is a fraction of that by subcutaneous injection. In the case of serious illness where serum therapy is required, this should not be withheld from fear of anaphylaxis. With regard to the possibility of there being a primary or *natural* supersensitiveness, inquiry should be made as to tendency to asthma or hay fever, or sensitiveness to the presence of horses in the vicinity, as these have been found to be associated conditions, and the existence of Graves' disease has been recorded as another. Then with regard to the *acquired* variety, information should be obtained regarding previous serum injections. The existence of supersensitiveness can be tested for by demonstrating skin allergy. A small quantity, say 0.05 to 0.2 c.c., of a 1/10 or 1/100 dilution of sterile horse serum with saline is injected by a hypodermic syringe into the dermis—not subcutaneously—a similar quantity of saline alone being injected into another site by way of control. The minute local swelling which results from the presence of the fluid soon passes off. But in the case of a positive reaction there occurs, usually within five to thirty minutes, an urticarial patch, which may be followed by a distinct vesicle and is often surrounded by an erythematous area, an inch or more in diameter. Alternatively one drop of 1/10–100 dilution of the serum may be allowed to fall into the conjunctival sac, a positive result is indicated by hyperæmia, swelling, itching, or pain coming on within one hour (which should be treated by instillation of adrenaline). Another method is to inject by the same route as that intended for the main dose 0.1 to 0.5 c.c. of a ten-fold dilution of the serum in sterile saline. If a reaction occurs within half to one hour in these tests the presence of supersensitiveness may be inferred. But a negative result, at least in the skin test, does not exclude the possibility of serious symptoms when the serum is administered. When a positive reaction is obtained, the attempt should be made to desensitise the patient, *i.e.* to produce anti-anaphylaxis, the procedure is to introduce initial small doses of serum and then gradually to increase them. Even then, care should be used in injecting the serum intravenously or intrathecally, as desensitisation in the human subject is uncertain.

The initial desensitising dose is 0.01 c.c. given subcutaneously, and this amount is doubled every half-hour. If no reaction follows the administration of 1 c.c., the subsequent doses are given intravenously, commencing with 0.1 c.c. and doubling the dose every half-hour till the total dose has been given. Where it is desired to inject the serum intrathecally the above procedure should be followed till an intravenous dose of 10 c.c. has been given, then the same amount may be injected intrathecally. Such a method however, takes a considerable number of hours and is not justifiable in a case of tetanus, where a large amount of serum should be given intravenously or intrathecally as soon as possible. In such circumstances the following method may be followed. 5 c.c. of the antiserum are diluted with 50 c.c. of normal salt solution. Of the mixture 1 c.c. is

injected intravenously; this is followed four minutes later by 3 c c, two minutes later by 10 c c, and two minutes later again by 25 c c. Then after ten to fifteen minutes the full dose may be given intravenously or intrathecally. The doses mentioned are most suitably given by the gravitation method of injecting serum intravenously or intrathecally, and the serum should be diluted with saline.

In all cases the administration of serum intravenously or intrathecally should be carried out slowly and with caution by the gravitation method, the serum being diluted with saline and warmed to about 37° C. If any anaphylactic symptoms appear, the administration must be temporarily stopped and then cautiously resumed. Adrenaline and atropine are the most efficient drugs, and a tourniquet may be applied above the site of injection. Anaphylaxis is sometimes a real danger, but when the necessity for the prompt treatment of tetanus is taken into account, the risks have been exaggerated. Repeated subcutaneous injections for preventive purposes of a few c c of serum are practically unattended by any danger. It may also be stated that in relation to anaphylaxis it is only the *amount of serum* and the *species* from which it is drawn which matter—the antitoxic value is not a factor. Accordingly it has been suggested that when antiserum is given solely with a view to prophylaxis, *e g* of diphtheria, it should be derived from another species such as the goat or ox, thus the development of super-sensitiveness to horse serum is avoided, in case later on a potent dose of antibody contained in the latter should be required for treatment.

CHAPTER IV

BACTERIA ASSOCIATED WITH ACUTE INFLAMMATION AND SUPPURATION—STAPHYLOCOCCI AND RELATED ORGANISMS · STREPTOCOCCI

A CONSIDERABLE number of species of bacteria have been found in acute inflammatory and suppurative conditions, and most of these have been proved to be causally related. But it must be recognised that although many organisms when experimentally introduced into the tissues are capable of producing acute inflammation and even pus formation, only certain species are characteristically associated with such lesions under natural conditions. These organisms are usually classed as *pyogenic*; certain of them attack practically any part of the body, *e.g.* staphylococci and streptococci, while others show a predilection for particular sites, *e.g.* pneumococcus, gonococcus, meningococcus.

Many experiments have been performed to determine whether suppuration can be produced in the absence of micro-organisms by various chemical substances, such as croton oil, nitrate of silver, turpentine, etc.—care being taken to ensure the absence of bacteria. It may be said that in certain animals and with () the pus being free from bacteria by the injection c

cyaneus, etc., and, according to Vaughan, bacterial protein *per se* may produce suppuration and necrosis of tissue. Further, the injection of certain substances into a serous cavity, *e.g.* aleuronat, peptone, sodium nucleate, etc., may produce within twenty-four hours an abundant exudate containing large numbers of polymorph leucocytes, and in this way such cells can be obtained unmixed with other blood cells for experimental studies of their immunological and other properties. However, the general statement may be made that practically all cases of true suppuration met with clinically are due to the action of living micro-organisms.

Ogston, who was one of the first to study the organisms responsible for suppuration (1881), found that those most frequently present were micrococci, of which some were arranged irregularly in clusters (staphylococci), whilst others formed chains (streptococci). He noted that the former were more common in circumscribed acute abscesses, the latter in spreading suppurative conditions. Rosenbach shortly afterwards (1884), by means of cultures, differentiated several varieties of micrococci, to which he gave special names. *e.g.* *Staphylococcus pyogenes aureus*, *Staphylococcus pyogenes albus*, *Streptococcus pyogenes*. Suppuration may be produced, however, by other species: *e.g.* *Micrococcus tetragenus*, pneumococcus, meningococcus, gonococcus, coliform bacilli, Friedlander's pneumobacillus, *Bacillus pyocyaneus*, *Bacillus proteus*, *Actinomyces*, *Bacillus mallei*, etc. Even the typhoid and paratyph () may be associated () of enteric fever ()

inflammatory and suppurative conditions. Various anaerobic bacteria are concerned in the production of an inflammation which is often associated with œdema, hæmorrhage, or necrosis.

Later investigations showed that the subject of the pathogenic action of bacteria is a very complex one, and that they may produce a variety of different general characters and local effects, and the usual suppurative processes, various inflammatory conditions, endocarditis, septicæmia, and pyæmia, require consideration. The following general statements may be made. In the first place, various organisms may produce substantially the same type of lesion, and not infrequently several species of organisms may be present together. In the second place, the same organism may produce widely varying results under different circumstances—at one time a local inflammation or abscess, at another multiple abscesses, or a general septicæmia. The principles on which this diversity in results depends have already been explained (Chapter II). Furthermore, there are conditions like acute pneumonia, epidemic meningitis, scarlatina, etc., which have practically the characters of specific diseases, and yet, as regards their essential pathology, belong to the same class.

The following are among the chief points in the pathology of these conditions. In *suppuration* the two main phenomena are—(a) a progressive emigration of leucocytes, chiefly of the polymorphonuclear (neutrophile) variety, and (b) a liquefaction or digestion of the supporting elements of the tissue along with necrosis of the cells of the part. The result is that the tissue affected becomes replaced by pus. A suppurative inflammation is thus to be distinguished on the one hand from an inflammation without destruction of tissue, and on the other from necrosis or death *en masse*, where the tissue is not liquefied, and leucocyte accumulation may be slight. When, however, suppuration takes place in a very dense fibrous tissue, liquefaction may be incomplete, and a portion of dead tissue or slough may remain in the centre, as is the case in boils. When suppuration occurs in a serous cavity the two chief factors are the progressive leucocytic accumulation and the disappearance of any fibrin which may be present. If the tissue defences overcome the infection before suppuration has taken place, regression of the lesions (resolution) occurs, this is commonly the case in lobar pneumonia. Again, when the site of infection is a mucous membrane, it frequently happens that a catarrhal inflammation with mucopurulent secretion results without abscess formation. When pyogenic streptococci infect the lymphatics of the skin a spreading inflammation without suppuration (erysipelas) is the rule.

The term *septicæmia* is applied to conditions in which the organisms multiply within the blood and give rise to symptoms of general poisoning, without, however, producing abscesses in the organs. The organisms are usually more numerous in the capillaries of internal organs than in the peripheral circulation, but by means of blood culture it has been shown that they can be detected in the peripheral blood. It should be remembered that bacteria may also be demonstrable in the blood as a result of their 'overflow' from an infective lesion in the tissues, and such blood infection—*bacteriæmia*—does not necessarily connote a true septicæmia or multiplication of organisms within the vascular system. The essential fact in *pyæmia*, in contrast with septicæmia, is the occurrence of multiple abscesses in internal organs and other parts of the body. In most of the cases of typical pyæmia, common in pre-antiseptic days, the starting-point of the disease was a septic wound with bacterial invasion of a vein, leading to thrombosis and secondary embolism. Multiple foci of suppuration may be produced, however, in other ways, as will be described below.

Mode of entrance and spread—primary infection. Many of the pyogenic organisms have a wide distribution, being present on the skin and mucous

membranes of healthy persons and also animals. Staphylococci and streptococci are constant commensals in the mouth, nose, and throat; and typical pyogenic streptococci may occur in the throat of apparently normal individuals, particularly in the tonsillar crypts. The entrance of these organisms into the deeper tissues when a surface lesion occurs, can be readily understood. Their pathogenic action will, of course, be favoured by any condition of depressed vitality. Though in normal conditions the blood is bacterium-free, the clinical and other evidence indicates that from time to time a certain number of organisms gain entrance to it from trifling lesions of the skin or mucous surfaces, the possibilities of entrance from the latter being especially numerous. In most cases they are inhibited and killed by the action of the plasma or phagocytes, and no lesion results. If, however, there be a local damage, they may settle in that tissue and produce suppuration, and from this other parts of the body may be infected. In some cases of multiple suppurations due to infection with staphylococci, only an apparently unimportant surface lesion is present; whilst in others no lesion can be found to explain the origin of the infection, the latter being termed *cryptogenetic*. It is usually impossible to decide whether in such cases the infection has been determined by an organism of high virulence or by abnormal susceptibility of the host.

The *paths of secondary infection* may be conveniently summarised thus: *First*, by lymphatics, in this way the lymphatic glands may be infected, and also serous sacs in relation to the organs where the primary lesion exists. *Second*, by natural channels, such as the ureters, the spread being generally associated with an inflammatory condition of the lining membrane. In this way the kidneys may be infected. *Third*, by the blood vessels: (a) by a few organisms gaining entrance to the blood from a local lesion, and settling in a favourable nidus or damaged tissue, the original site of infection often being the thrombus of the thrombus vein producing a lesion in the alimentary canal, the condition being known as 'pyelephlebitis suppurativa'.

THE STAPHYLOCOCCI

Staphylococcus pyogenes aureus (*Micrococcus pyogenes* var. *aureus*).
MICROSCOPIC CHARACTERS. This organism is a spherical coccus, which tends to grow in irregular clusters or masses (Figs 5, 6), since after a coccus has divided the subsequent planes of division of the daughter cells bear no fixed relation to each other; single organisms, pairs, tetrads, or short chains may also be observed. The opposed surfaces of adjacent cocci may be flattened. From young agar cultures the cocci are very uniform in size, 0.8 to 0.9 μ in diameter, in pus the size is variable, larger forms occurring in abscesses. It is non-capsulate, grows in both cultures and in nature, retains the colour in Gram's method; but with some strains older cultures may show a number of decolorised cocci.

CULTURAL CHARACTERS. Growth occurs under aerobic conditions, but the organism is also a facultative anaerobe. It grows readily in all the ordinary media at room temperature, though much more readily at the temperature of the body. The temperature range for growth is 10° to 42° C., the optimum temperature about 37° C. On agar, a stroke culture forms a

line of abundant yellowish growth, with smooth shining surface, well developed after twenty-four hours at 37°C ; it readily yields a uniform suspension in water. Later it becomes bright orange in colour, and resembles



FIG 5 Staphylococci in pus. Stained by Gram's method $\times 1,000$

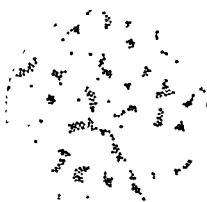


FIG 6 *Staphylococcus aureus*, young culture on agar, showing clumps of cocci. Stained by Gram's method $\times 1,000$

a streak of oil paint. Single colonies on the surface of agar are circular discs of similar appearance, which may reach 3 to 4 mm after twenty-four to forty-eight hours. In a primary culture from pus, some or all of the colonies after twenty-four hours at 37°C may be much smaller, also pigment may be absent, so that to the naked eye they resemble a growth of streptococci (*vide infra*). (But if the growth is caused to heap up into a mass by drawing a platinum loop along the surface of an agar slope culture, then the pigmentation often becomes apparent.) Pigment production is manifest when the organism is growing in the presence of oxygen, and is most pronounced in cultures kept at room temperature and exposed to light. The pigment is a hypochrome and is stated to be allied to carotene. On *blood agar* a clear zone of hæmolysis is noted round the colonies (*vide infra*, hæmolysin). In *stab cultures in gelatin* a streak of growth is visible on the day after inoculation, and on the second or third day liquefaction commences at the top. As liquefaction proceeds, the growth falls to the bottom as a flocculent deposit, which soon assumes an orange-yellow colour, while a yellowish film may form on the surface, the fluid portion still remaining turbid. Ultimately liquefaction extends out to the wall of the tube (Fig 7). In *gelatin plates* colonies may be seen with the low power of the microscope in twenty-four hours as little spheres, somewhat granular on the surface and of brownish colour. On the second day they are visible to the naked eye as whitish-yellow points, which in typical strains afterwards become more distinctly yellow. Liquefaction occurs around these, and small cups are formed, at the bottom of which the colonies form yellowish masses. On *coagulated serum*, pigment production is usually marked and often slow liquefaction or softening of the medium occurs. On *potato* the organism grows well at ordinary temperature, forming a somewhat abundant layer of



FIG 7 Stab culture of *Staphylococcus aureus* in gelatin — 17 days old, showing liquefaction of the medium and characters of growth. Natural size

orange colour. In *broth* it produces usually a uniform turbidity, which afterwards settles to the bottom as an abundant deposit and assumes a brownish-yellow tint; some strains form a ropy or mucoid growth. It grows readily in *milk* acidifying the medium and often producing coagulation. On *MacConkey's agar* the colonies are small and at first characteristically tinted yellowish-pink, later red.

BIOCHEMICAL REACTIONS. Various carbohydrates, *e.g.* glucose, lactose, saccharose, mannitol, are fermented with formation of acid but no gas.

Coagulase. Following the early observations of Loeb and Much, it has been found that the power to coagulate citrated or oxalated blood plasma of rabbit or man is one of the most constant properties of pathogenic staphylococci. Coagulase may be demonstrated directly in purulent material even when there is a mixed infection or where staphylococci are too scanty to be demonstrated microscopically. This direct test is reliable and also saves the time required for isolation of a pure culture (Lominski *et al.*). Coagulase is relatively thermostable, as it partially withstands heating at 100° C. Its presence can be demonstrated readily in sterile filtrates of cultures in a fluid medium provided the latter contains plasma (Lominski). Fermentable carbohydrate in the medium interferes with demonstration of coagulase.

Method of test for a culture (Fisk) To 0.5 c.c. of a 1:10 dilution in saline of citrated human plasma in a small test tube add 0.125 c.c. (5 drops from a capillary pipette) of a pure eighteen to twenty-four hours' broth culture. Keep at 37° C. for three hours and then at room temperature overnight. Coagulum forms usually in half to one hour, but three to eighteen hours may be necessary. The controls required are (a) a tube of the same diluted plasma inoculated with a known coagulase-positive culture and (b) a tube of uninoculated plasma. The plasma remains usable for several weeks if kept undiluted and sterile.

Direct test Two or three loopfuls of the material are added to 0.5 c.c. of a mixture of plasma 1 vol., saline 3 vols., broth (not digest) 1 vol., and heparin 5 T.U. per c.c. If a swab moistened with the material is used, it is well squeezed out by dipping into the diluted plasma and pressing repeatedly against the wall of the tube. The tube is kept at 37° C. and the test is read after half, one, six, and twelve hours. Controls are set up as above. The early readings must not be omitted, since a coagulum which forms within several hours may liquefy later if proteolytic organisms are present.

VARIANTS Variation as regards pigmentation, consistence, and 'smoothness' or 'roughness' of colonies has been observed. Thus Bigger *et al.* have described the appearance of a white variant in old cultures of the *aureus* type and various observers have agreed that the *albus* type may be derived from the *aureus*.

Staphylococcus pyogenes albus (*Micrococcus pyogenes* var. *albus*) is similar in general characters to *Staph. aureus*, with the exception that its growth on all the media is white. It may be said that in general the *albus* type of staphylococcus is weaker in its biochemical, toxic, and pathogenic properties than *Staph. aureus*. A similar organism, called by Welch *Staphylococcus epidermidis albus* is practically always present on the skin and often on the conjunctiva, it liquefies gelatin slowly, does not ferment mannitol, is non-hæmolytic, and does not form coagulase.

Other Varieties. *Staph. citreus*, which is seldom met with in suppurative lesions, yields lemon-yellow growths. It is usually less virulent than the other two. *Staph. cereus flavus* and *Staph. cereus albus*, so designated in virtue of their wax-like growth and the coloration produced, do not liquefy gelatin. It is doubtful if they are pathogenic. *Staphylococcus ascoformans* is a type of staphylococcus similar to *Staph. aureus* and associated with equine 'botryomycosis'. In the lesions it forms large clusters, and the cocci are capsulate. Similar appearances have been met with rarely in human lesions (Berger *et al.*, Fink).

VIABILITY. Staphylococci have considerable tenacity of life outside the body, and withstand drying even for several weeks. Laboratory cultures remain viable for months. The thermal death-point is approximately 62°C , but some strains resist higher temperatures, even 70°C for half an hour. Crystal violet is very inhibitory to staphylococci, growth seldom occurring on agar containing over 1 : 300,000 of the dye.

Toxins. As indicated above, staphylococci, particularly the pathogenic *aureus* type, produce hæmolytic toxins, in addition, there are other manifestations of toxic action. Culture-filtrates when injected intravenously in rabbits may rapidly lead to death, and the toxin acts similarly on intraperitoneal injection, mice are also susceptible. When injected in smaller quantity into the skin of rabbits or guinea-pigs a necrotising effect is produced which is said to be due to 'necrotoxin'. Filtrates may also possess the power of killing leucocytes, and the active principle (as in the case of other bacteria yielding a similar product) has been designated 'leucocidin'. In addition there is the 'enterotoxin' which is formed by some strains. It is not certain whether coagulase is of the nature of a toxin (*vide infra*).

Hæmolysin. A soluble hæmolysin, which has the general properties of an exotoxin, can be demonstrated in filtrates of suitable broth cultures or by suspending a twenty-four hours' agar culture in a small amount of saline and then removing the organisms from the fluid by centrifuging at high speed (Bigger *et al.*) This product is usually inactivated at 65°C although it may be less damaged at 80° to 100°C . The production of hæmolysis by cultures growing on blood agar, however, is not necessarily associated with formation of soluble hæmolysin. A neutralising antitoxin can be obtained by immunising animals with hæmolysin. Glenny and Stevens have shown that some strains of staphylococci produce two different hæmolysins (designated α and β) for which separate antitoxins can be obtained. The α hæmolysin is characterised by rapid action at 37°C . on rabbit, ox, and sheep red cells, human and horse erythrocytes are relatively resistant. The β hæmolysin is characterised by its continuous and progressive action on sheep red cells at room temperature after preliminary incubation at 37°C ., ox and human red cells are also sensitive. Strains of staphylococci from animal lesions, e.g. bovine mastitis, may yield the β hæmolysin (Minnett). Another antigenically distinct hæmolysin (γ) which acts on the red cells of the rabbit, etc., has been described (Smith *et al.*).

Lethal action. After an intravenous injection death may occur in a few minutes or not for twenty-four hours. The chief effects are direct action on the heart and the capillaries of the lungs. Guinea-pigs are less susceptible than rabbits. In cats, vomiting is an early symptom, the experimental disease resembling that in the children at Bundaberg (*vide infra*). Preformed histamine contained in the cultures has been excluded as the cause of the symptoms. According to Burky the lethal factor is stable as compared with the lability of the hæmolysin.

Necrotoxin or dermoxin. The necrotising effect of a culture-filtrate injected intradermally frequently parallels its lethal action intravenously.

Leucocidin can be demonstrated by the methylene blue reduction test. Rabbit leucocytes are obtained by intrapleural injection of aleuronat, washed free from serum and suspended in normal saline, these cells in the healthy state reduce methylene blue under partially anaerobic conditions, but lose this property after they have been acted on by leucocidin. J. Wright has found that this toxin is identical in thermostability (at 10°C) with the α hæmolysin and differs from the β hæmolysin, which is stable at 56°C . Destructive action by staphylococcal filtrates towards human leucocytes

as observed microscopically (Panton and Valentine) is apparently due to a different leucocidal factor. The latter also annuls the power of rabbit leucocytes to reduce methylene blue; but the changes in the leucocytes differ microscopically from those produced by a hæmolysin (Proom).

Enterotoxin This toxic factor is apparently formed by only a minority of strains (Dolman). Laboratory animals are not readily susceptible to the toxin when introduced into the stomach, although symptoms of poisoning (*e.g.* gastro-enteritis) can be produced when the acidity of the stomach contents is neutralised at the same time (Borthwick). Woolpert and Dack obtained results with culture-filtrates in *rhesus* monkeys similar to those in man. The toxin is inactivated by even a slight degree of acid or alkali, but may resist boiling for some time, not being completely inactivated after thirty minutes. For demonstrating enterotoxin the intraperitoneal injection of boiled filtrates into young cats (500 grms. weight) has been recommended (Dolman and Wilson). Vomiting sets in after one quarter to one and a half hours and there is also diarrhoea. According to Fulton this test is not reliable, since the symptoms are evidence of peritoneal irritation, and do not parallel the enterotoxin effect produced in man by the same materials when taken by mouth.

The question arises whether these various effects are due to a single toxin or to several different toxic substances; evidence of two sorts has been obtained. (1) As already mentioned, it has been shown in the case of hæmolytic toxins, that several different kinds exist, which are characterised by the conditions under which they act, the species of blood attacked, and the possession of distinct antigens. Also, filtrates have been investigated which possessed in marked degree certain of the toxic properties while lacking others *e.g.* powerfully hæmolytic but with no necrotoxin, or strongly necrotoxic but weak as regards lethal action when injected intravenously in rabbits, (2) On the other hand, a hæmolytic toxin (β) can be titrated by its lethal action for rabbits or the effect produced on the skin of the guinea-pig, as well as by its action on sheep red cells (Smith and Price). Also, the different effects are neutralised in parallel by an antitoxic serum, as shown in the case of a hæmolysin (Levine). Accordingly, the evidence, though incomplete, indicates that there are diverse toxins, but that, at least in certain cases, one toxic component may damage several different tissues. It may be said that strains vary quantitatively in the toxicity of their products and there is no strict quantitative correlation as regards the production of the different factors. There is also no definite correlation between the pathogenicity or virulence of strains and the degree of toxicity.

Fibrinolysin is produced by certain pathogenic staphylococci, especially those derived from lesions in man. Its action is manifested by solution of the plasma-clot occurring in the course of several days.

Hyaluronidase is formed by strains of staphylococci which show marked invasive properties.

Preparation of toxins Walbum's medium is suitable for the production of the staphylococcal toxins. It consists of an ox heart extract in which are incorporated 0.5 per cent Witte's peptone, 0.2 per cent potassium dihydrogen phosphate and 0.03 per cent magnesium sulphate (adjusted to a pH of 6.8). The growth is obtained in an atmosphere of 20 per cent carbon dioxide. The following method for preparing staphylococcal toxin has been used by Parish and Clark. 2 per cent nutrient agar is diluted with Parker's buffered broth (equal parts of M/15 potassium dihydrogen phosphate solution—pH 7.4—and ordinary sugar-free meat infusion broth containing 4 per cent Witte's peptone) until the concentration of agar is 0.8 per cent. The medium is then sloped in suitable containers. The culture is grown in an atmosphere of 25 per cent

carbon dioxide After forty-eight hours' growth more buffered broth is added to the culture, and growth is continued for twenty-four to seventy-two hours as before in an atmosphere of 25 per cent. carbon dioxide Gladstone has obtained powerful hæmolytic by cultivation in a chemically defined medium

The occurrence of endotoxic substances has been referred to by various workers, but those are apparently relatively weak in action and formed in small quantity

Toxoid Staphylococcal toxin, like other exotoxins, can be changed to toxoid by appropriate treatment with formaldehyde and in such non-toxic form can conveniently be used as an antigen for immunisation

Habitat: Lesions in Man and Domestic Animals. Staphylococci occur as commensal organisms on the skin, in the mouth, throat, auditory meatus, nose, conjunctiva, and in human and animal milk, the prevalent type being *Staph. albus*, although *Staph. aureus* occurs also, especially in the nose They may also be present in the anterior urethra, stomach, and occasionally in the intestine Consequently these organisms are met with in the air, in dust on clothing, etc. Staphylococci have been found in an apparently latent state in the tonsils and in lymphatic glands

Lesions in the human subject Staphylococci are the most common causal agents in skin, carbuncles, abscesses, etc. in new-born caused by suppurating wounds and burns, catarrhs of mucous surfaces, urinary sepsis, acute bacterial endocarditis and pyæmic conditions They are occasionally the cause of septicæmia and pneumonia Certain cases of acute food poisoning have been traced to staphylococcal toxin produced by these organisms growing in the particular article of food (Jordan) The illness has usually been an acute gastro-enteritis developing after an incubation period of two to four hours and passing off in twenty-four hours Milk from cows affected with staphylococcal mastitis has been responsible in one outbreak (Crabtree and Litterer). Also, the organisms producing the condition have on occasion been traced to human sources, the foods usually responsible being cooked meats and 'creams' which have been subjected to handling Jordan and Hall reproduced the disease in human volunteers by administering *per os* small quantities of culture-filtrates of staphylococci isolated from incriminated food-stuffs.

Infections of domestic animals. Staphylococci are commensals of the skin and mucous membranes of animals, but are less frequently associated with disease than in the human subject They occur, however, in inflammatory and suppurative lesions either as primary or secondary infecting organisms, e.g. wound suppuration, fistulæ, localised abscesses etc Cases of bovine mastitis, both acute and chronic, are not infrequently due to *Staph. aureus* and the acute infection may assume a markedly destructive or even gangrenous form. Another condition due to *Staph. aureus* is pyæmia of lambs associated with tick infestation, and apparently the result of a primary infection of the tick-bite In equines a staphylococcus is associated with the chronic suppurative disease often described as 'botryomycosis', in the lesions the cocci may not unlike the 'gram designated *Staph. as.* distinguished from *Staph. aureus*.

Experimental Inoculation. It may be stated at the outset that the occurrence of suppuration depends upon the number of organisms introduced into

the tissues, the dose necessary varying not only in different animals, but also in different parts of the same animal—a smaller number producing suppuration in the anterior chamber of the eye, for example, than in the peritoneum. The virulence of the organism also may vary, with correspondingly variable results on experimental inoculation. Rabbits are highly susceptible and have been used as a rule. *Staph. aureus*, when injected subcutaneously in suitable numbers, produces an acute local inflammation, which is usually followed by suppuration. If a large dose is injected, the cocci may enter the blood stream in sufficient numbers to

Intravenous injection

used. If

quantity

some become

parts and produce minute abscesses. These are most common in the kidneys, where they occur both in the cortex and medulla as minute yellowish areas surrounded by zones of intense congestion and hæmorrhage. Similar small abscesses may be produced in the heart wall, lungs, liver, under the periosteum and in the interior of bones, and occasionally in the striped muscles. Very rarely indeed do the cocci settle on the healthy valves of the heart. If, however, when the organisms are injected into the blood, there be any traumatism of a valve, or of any other part of the body, they show a special tendency to settle at these weakened points. Multiple abscesses in the bones and under the periosteum may follow intravenous injection, especially when young animals are used. But the processes do not spread in the same way as in the natural disease, acute osteomyelitis and periostitis in the human subject. Presumably in the latter there is some local susceptibility, which enables the few organisms which have reached the part by the blood to settle and multiply. This is supported by the finding that if a bone be experimentally injured, e.g. by actual fracture or by stripping off the periosteum before the organisms are injected, then a much more extensive suppuration occurs at the injured part.

Mice vary in susceptibility both individually and also to different strains. Intraperitoneal injection of 0.1 c.c. of a twenty-four hours' broth culture may cause death in a few hours, apparently from toxæmia; or septicæmia or pyæmia may prove fatal in twenty-four to forty-eight hours. Again, with the same culture other animals, after being very ill, recover, but often develop suppuration in the needle track later, from which they may die in some weeks.

Experiments on the human subject have also proved the pyogenic properties of these organisms. Garré inoculated scratches near the root of his finger-nail with a pure culture, a small cutaneous pustule resulting; and by rubbing a culture over the skin of the forearm he caused a carbuncular condition which healed only after some weeks. Confirmatory experiments of this nature were made by others. Inoculation with living *Staph. aureus* occurred in a series of children at Bundaberg, Australia, who received an injection of 2 or 4 minims of an accidentally contaminated preparation of diphtheria toxin-antitoxin prophylactic which contained no antiseptic. Eighteen out of the twenty-one fell ill in five to eight hours, in most cases with an attack of vomiting and fever, this being followed by severe circulatory collapse and death in twelve, which occurred after fifteen to thirty-four hours. The survivors developed abscesses at the site of injection (Rep. Roy. Comm.)

The effects produced by the experimental injection of the staphylococcal toxins have been dealt with above.

Mode of Pathogenic Action. The events in severe acute suppuration indicate that the greatest effect occurs in the vicinity of the organisms, which are usually abundant. But leucocytosis, fever, and general symptoms point also to the circulation of poisonous products. The question arises as to how far the various phenomena which have been investigated experimentally explain the natural disease picture. Killed cultures of staphylococci do not cause any marked local lesion unless when injected in large doses, *i.e.* there does not appear to be a marked endotoxic effect, although it is likely that products are formed in the tissues which do not appear *in vitro*. On the other hand, the soluble toxins can cause both local and general effects in many respects similar to those met with clinically (Burnet). Further, according to Menkin the damaged tissues yield a crystalline nitrogenous substance, leukotaxine, which is probably a relatively simple polypeptide, this causes increased permeability of the capillaries and also emigration of leucocytes, the latter being interfered with later by local acidosis due to disturbance of the metabolism of the tissue carbohydrates. According to Lyons, young organisms, in the 'invasive' phase, possess capsules which protect them from ingestion and destruction by leucocytes.

In contrast to what is seen with *Streptococcus pyogenes*, a feature of staphylococcal infection is the tendency to form localised collections of pus. The agglutinating action of normal serum on the staphylococci may be one factor tending to limit their spread. Also it has been found experimentally that staphylococci quickly cause the lymphatics draining the site of infection to become impervious, whereas in streptococcal infection they continue to drain the area for a long period (Menkin, 1935). Menkin and Walston have endeavoured to ascertain whether the fibrinous thromboses observed in lymphatic vessels following the intradermal injection in rabbits of filtered cultures is due to coagulase. Their findings indicate, however, that the rapid obstruction of lymphatic channels is produced by the necrotoxin and not by coagulase. The rôle of coagulase with respect to pathogenic action is indicated, however, by the findings of Wilson Smith and his associates, that the presence of coagulable plasma inhibits the phagocytosis of coagulase-producing staphylococci *in vivo*, as well as *in vitro*, and so is important in the initiation and establishment of infection.

The tendency for persistence of staphylococci at old sites of infection with recrudescence of the disease later on indicates the advisability of removing such foci. In severe infections in man, according to Butler and Valentine, it is important for prognosis to distinguish between the toxic and the invasive features. This may be done by repeated quantitative blood cultures. Where 30 to 100 or more colonies are present per c.c. of blood, or where their number progressively increases, death is the rule, whereas when the number of organisms is smaller, even although general symptoms are severe, recovery is likely.

Immunity and Serological Reactions. Animals can be immunised experimentally by repeated and graded doses of killed or living organisms, and various specific antibody reactions can be demonstrated with immune sera, *e.g.* opsonic action, agglutination, precipitin reaction, complement fixation, but immune bactericidal antibody is not developed in such sera. Staphylococcal vaccines have been extensively used for the prophylaxis and treatment of recurrent and chronic infections, *e.g.* furunculosis, but the results recorded by various observers are somewhat contradictory.

Filtrates containing the toxins of staphylococci lead to the development

of antitoxins *in vivo* and the same is true of toxoids. Rabbits immunised with staphylococcal toxoid exhibit a higher degree of resistance to living cultures of the organisms (when administered subcutaneously or intravenously) than animals which have been injected with washed vaccines (Parish *et al.*; Downie; Smith); nevertheless local lesions may develop later. Antitoxic serum from immunised horses has been used therapeutically in human infections. Although it would appear to be promising in acute and toxic cases, its value has not yet been clearly established. Immunisation with toxoid has been employed with some success in the treatment of localised staphylococcal lesions, e.g. furunculosis, sycosis. According to Dolman clinical improvement is accompanied by a rise in the antitoxic titre of the patient's serum. Murray has estimated the average antitoxic value of human blood and compared it with that following immunisation with toxoid; a considerable increase was observed. The results of J. F. Smith, however, in a controlled series of cases of furunculosis indicated that the content in antibody of the patient's blood is a relatively unimportant factor in this condition; a similar conclusion was reached by Hite *et al.* in chronic osteomyelitis. Forssman also has emphasised that an animal's immunity to inoculation with live staphylococci need not parallel the antitoxin content of its blood. Antihæmolyisin has been demonstrated in normal human blood (Bryce and Burnet) and this tends to increase in infected subjects. Antileucocidin has also been shown to occur. Human sera frequently contain a substance inhibitory to staphylocoagulase (Lominski and Roberts). Its incidence appears to be higher in healthy people than in those with severe staphylococcal infections.

A cutaneous reaction in the human subject analogous to the Dick and Schick reactions may be elicited by intradermal injection of staphylococcal filtrate, but the significance of positive and negative results in this test has not yet been fully assessed.

As in various other bacterial groups, complex specific carbohydrates enter into the antigenic composition of the staphylococci (Julianelle and Wiegand, Hoffstadt and Clark), and these substances when isolated from the bacterial substance yield specific reactions with antisera. The relationship of specific carbohydrates to other characters of the organisms requires further study. Verwey has found, however, that protein antigens are also responsible for precipitin reactions.

Classification of Staphylococci. It has been seen that cultures of staphylococci recovered as the sole organisms from acute inflammatory or suppurative lesions, or from the blood in cases of pyæmia or septicæmia, i.e. pathogenic strains, usually produce in varying degree golden yellow pigment, coagulase, and soluble toxins of which hæmolyisin is the most easily demonstrated; they also ferment mannitol and liquefy gelatin. Less commonly the cultures are white, the other characters being the same. These correspond respectively with *Staph. aureus* and *Staph. albus*, it being recognised that the one merges into the other, accordingly they may be classed together as *Staph. pyogenes* (Fairbrother). According to the presence of pigment and coagulase are the chief characters.

that the most constant characters of the organism may be used to distinguish between the two species. Staphylococci which are recovered under other circumstances, e.g. from the intact skin, normal or inflamed mucous membranes, the air, etc., are usually classed as 'potential pathogens' if they possess the above characters of *Staph. pyogenes*. But nothing can be inferred with certainty from those

properties, including production of toxin *in vitro*, regarding the degree of their virulence. Also, enterotoxin-producing strains are not distinguishable by any other features.

when brought into

ical characteristics in order to distinguish groups and types, especially with a view to epidemiological investigations for tracing the source of outbreaks of staphylococcal infection. Direct agglutination has not proved useful, since antisera tend to agglutinate various strains; but sera which have been absorbed with homologous strains are deprived of agglutinin for the latter, whereas absorption with heterologous strains does not remove the power to agglutinate the homologous. Hence, by the use of such absorbed sera, divided *Staph. pyogenes* (coloured or

growths and fermented mannitol were classed serologically as *Staph. epidermidis*. Cowan, by means of a simple slide agglutination reaction with absorbed antisera, has divided two-thirds of the strains of *Staph. pyogenes* into three types (I, II, III), saprophytic strains do not belong to these types. According to Christie *et al.*, agglutination reactions show that staphylococci from furuncles and severe lesions, e.g. osteomyelitis, usually differ from those found in the nose and throat in health, also the former are more complex antigenically. By means of the precipitin reaction several pathogenic groups can be broadly distinguished from non-pathogens. The latter tend to be antigenically heterogeneous.

By typing according to their susceptibility to different preparations of bacteriophage, Wilson and Atkinson have recognised twenty-one types or subtypes of staphylococci.

Identification of sources of infection. The above-mentioned methods of agglutination and phage typing have been used for tracing sources of infection, especially in epidemic outbreaks of staphylococcal diseases in institutions, etc., e.g. wound infections, puerperal mastitis, pemphigus neonatorum, and food-poisoning. It would appear from the findings that contamination from nasal or throat carriers or actual staphylococcal lesions of the skin is frequently responsible for originating such outbreaks. Knott *et al.* recorded success in preventing outbreaks of infection in maternity wards by continuous bacteriological control, with exclusion of all carriers of 'invasive' types of staphylococci among patients and staff. In the case of food-poisoning due to milk or cheese, staphylococcal mastitis of cows or goats may be the source.

MICROCOCCUS TETRAGENUS (GAFFKYI TETRAGENA)

This organism, first described by Gaffky, divides in two planes at right angles to one another and is thus generally found in the tissues in tetrads (Fig. 8), which are often seen to be surrounded by a capsule. The cocci measure about 0.6 to 0.8 μ in diameter. They stain readily with all the ordinary stains, and also retain the stain in Gram's method. They grow readily in all the media at room temperature. In a stab culture in gelatin a fairly thick whitish line forms along the track of the wire, while on the surface there is a thick rounded disk of whitish colour, the gelatin is not liquefied. On the

150 BACTERIA ASSOCIATED WITH ACUTE INFLAMMATION

surface of agar and of potato the growth is an abundant moist, white layer. Cultures may have a viscid character.

Experimental inoculation. White mice are exceedingly susceptible to this organism when recently isolated. Subcutaneous injection is followed by a general septicæmia, large numbers of organisms being found in the blood throughout the body. Guinea-pigs are less susceptible; sometimes only a local abscess with a good deal of necrotic change results; sometimes there is also septicæmia.

M. tetragenus may occur as a commensal on the mucous membrane of the upper respiratory passages; it has also been found on the skin. It is often found in suppurations in the region of the mouth or in the neck, e.g. dental abscess, and also occurs in various lesions of the respiratory tract, in phthisical cavities, abscesses in the lungs, etc. Sometimes it is present alone, and probably has a pyogenic action in the human subject under certain conditions. In most cases it is associated with other organisms. Cases of general infection along with pneumonic symptoms have been recorded, the organism having been isolated from the blood; recovery was the rule. Cases of pyæmia have also been described in which this organism was found in a state of purity in the pus in various situations.




FIG 8 *Micrococcus tetragenus*; young culture on agar, showing tetrads Stained with carbol-fuchsin $\times 1,000$

OTHER MICROCOCCI RELATED TO THE STAPHYLOCOCCI

There exist numerous species of micrococci, some closely resembling staphylococci in microscopic characters and in the appearance of cultures, others in which the cocci are grouped as tetrads or in packets. Often the individual cocci are much larger than pathogenic staphylococci. Their cultures tend to be pigmented—lemon-yellow, orange, or red—and often they grow better at 25° C. than at 37° C. Generally they are Gram-positive. Many of them occur in air or water and so they are met with frequently as contaminants; but they are rarely, if ever, associated with pathogenic processes. It appears that they do not possess the characters usually associated with the pyogenic staphylococci, namely, production of coagulase or soluble hæmolyisin and liquefaction of gelatin. Attempts have been made to distinguish them by means of fermentation and other reactions in cultures, but without much success. The following are mentioned as of common occurrence, *M. luteus* and *M. ureæ*. *M. luteus* produces lemon-yellow growths, the cocci may be arranged in clusters (*M. lyso-deikticus* would appear to be a closely related species) or in packets (*Sarcina lutea*). *M. ureæ*, isolated from decomposing urine, produces ammonium carbonate from urea; its cultures are white.

THE STREPTOCOCCI

These organisms form a large and somewhat heterogeneous group, and though they present many characters in common, their taxonomy still presents certain problems from both the medical and general biological standpoints. The usual type found in suppurative conditions of the human

subject is *Streptococcus pyogenes*, the classical species described by Rosenbach. The biological characters and toxigenic properties of this organism will be considered first, as a basis for the study of the whole group of streptococci, their classification and relationships to disease. The general characters of *S. pyogenes* also exemplify many of the basic features of the group.

Streptococcus pyogenes. MICROSCOPIC CHARACTERS. This organism is a coccus, usually spherical, 0.7 to 1 μ in diameter, and occurring in chains composed generally of ten or more individuals (Figs. 9, 10). The chains vary in length, however, depending largely on the environment. As division may take place in many of the cocci at the same time, the appearance of a chain of diplococci is often met with. When microscopic preparations are made from cultures on solid media, chain formation may not be observed; in fluid media, however, chains are usually well developed. Studied by means of the electron microscope at very high magnifications, the streptococcal cells appear to have rigid outer membranes whose continuity explains the characteristic chain formation (Mudd and Lackman). In young



FIG. 9. Streptococci in acute supuration. Stained by Gram's method $\times 1,000$.

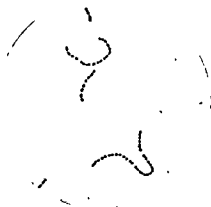


FIG. 10. *Streptococcus pyogenes*, young culture showing chains of cocci. Stained by Gram's method $\times 1,000$.

cultures the cocci are fairly uniform in size, but after a time they present considerable variation, many enlarging to twice their normal diameter. These are to be regarded as involution forms. The organism is non-motile and non-sporing. It stains readily with basic dyes and is Gram-positive. This reaction, however, is not so marked as in the staphylococci, and individual cocci may show only partial Gram-positive staining or even react negatively. By relief staining, virulent strains can be shown to possess capsules, but these are a marked feature only during the active phase of growth in a serum medium. The capsular material consists mainly of a polysaccharide, hyaluronic acid. After capsules have formed in a young culture they may undergo disintegration and this is apparently due to enzyme action. There is also evidence that the enzyme hyaluronidase, the 'spreading factor' referred to later in relation to the pathogenic action of the organism, affects capsule formation, thus, strains showing capsules fail to produce hyaluronidase while non-capsulate strains yield it (Morison, McClean).

CULTURAL CHARACTERS. In artificial culture *S. pyogenes* grows less abundantly than the staphylococci, and also dies more readily, being in most respects a more delicate organism. It grows aerobically or as a facultative

anaerobe on the ordinary culture media, *e.g.* nutrient agar, the optimum temperature being about 37° C. Only slight growth occurs at room temperature and no growth is obtained at 10° C. Growth is arrested between 43° and 45° C. The optimum pH is about 7.5; distinctly alkaline media, *e.g.* at pH 9.6, are inhibitory. The addition of blood or serum to the medium enhances growth, and blood agar is a convenient medium for routine cultivation (*vide infra*). The addition of 0.5 per cent. glucose also promotes growth.

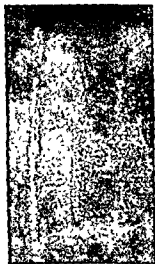


FIG. 11 Culture of *Streptococcus pyogenes* on an agar plate, showing numerous colonies—three successive strokes—Twenty-four hours' growth. Natural size.

On ordinary agar medium, growth takes place in the form of small greyish-white circular disks of semi-opaque appearance, which tend to remain separate (Fig. 11). The separate colonies remain small, not exceeding 1 mm. in diameter after twenty-four hours' growth. Under a low power of the microscope they have a granular appearance. Cultures on agar kept at the body temperature may often be dead after ten days. On blood agar, the colonies are larger (1 to 2 mm.) and are surrounded by a clear zone of laking or hæmolysis due to the hæmolysin produced by the organism. This presents a striking cultural character, and *S. pyogenes* and other streptococci showing this character have been designated 'hæmolytic

streptococci'. Hæmolysis occurs most readily under partially anaerobic conditions.

The colony appearances of strains are subject to a certain amount of variation, more manifest after growth has continued for some days. Variants can be separated which differ in the 'roughness' or 'smoothness'—three types: (1) opaque, flat, rough, and centre and thin translucent border, and there was no exact correlation between virulence and colony form the 'rough' varieties were generally the more virulent and were the usual forms found in pathological conditions. Todd (1927, 1930) described two colony types, 'matt' and 'glossy' respectively, the former either virulent or attenuated, the latter always attenuated. Dawson *et al.* in further studies of colony variants recognised four types, described as 'mucoid', 'matt', 'smooth', and 'rough', the 'matt' form being similar to that described by Todd and occupying a position intermediate between the mucoid and smooth forms. The mucoid, smooth and rough variants seem to be analogous to the corresponding phases of other bacteria.

In nutrient gelatin (if growth occurs at room temperature) a stab culture shows about the second day a thin line, which in its subsequent growth is formed of a row of minute rounded colonies of whitish colour; these may be separate at the lower part of the stab. They do not usually exceed the size of a small pin's head, this size being reached about the fifth or sixth day. The growth does not spread on the surface, and no liquefaction of the medium occurs. In broth, growth forms minute granules which afterwards fall to the bottom. The appearance in broth, however, presents variations, and if strains are repeatedly subcultured in this medium the growth becomes more uniformly turbid and less granular. More abundant growths can be obtained in a meat-digest broth than in meat-infusion medium.

BIOCHEMICAL REACTIONS. In milk a strongly acid reaction is produced, but no clotting of the medium. This organism ferments with acid production glucose, lactose, saccharose, salicin, and trehalose; mannitol is not usually

fermented, there is no fermentation of sorbitol, inulin, or raffinose, but different strains vary as regards their fermentative reactions

Further reference will be made to the characters of *S. pyogenes* in connection with the group of streptococci as a whole

VIABILITY. Cultures on ordinary media die in ten to fourteen days at room temperature, but survive much longer if kept at low temperatures. The organisms can be kept alive and virulent if rapidly dried at a low temperature and maintained in this state. A convenient method for preserving the viability of laboratory cultures, is to grow the organisms in a cooked-meat medium and then keep the culture at 0° C. Freezing below -15° C. of a culture in broth *plus* blood or boiled blood also preserves the virulence. The thermal death point is 54° to 55° C. *S. pyogenes* is fairly resistant to natural desiccation and remains viable for considerable periods in dust.

Toxins. As stated above, *S. pyogenes* has a distinct hæmolytic action, and this is due to the production of a diffusible toxin. The amount of hæmolysin formed varies greatly in the case of different strains and also according to the medium used. McLeod recommends a medium composed of 20 per cent horse serum and 80 per cent peptone broth. In this medium the maximum formation of hæmolysin is reached in about eighteen hours, and thereafter a diminution occurs. Hæmolysin can be demonstrated readily by cultivating the organism in serum broth for six to fifteen hours and then adding varying quantities (from 0.1 c.c. to 1 c.c.) of the culture to 0.5 c.c. of a 5 per cent suspension of washed ox red blood corpuscles. The mixtures are incubated at 37° C. for one and a half hours and hæmolysis, if present, can then be determined.

It has been shown that *S. pyogenes* produces two distinct hæmolysins (see Todd, 1932, 1942). One of these (designated 'O') is oxygen-labile and undergoes reversible oxidation; it is produced in large amount in a suitable broth medium *without serum*. Immunisation of animals yields a specific anti-hæmolysin. The other (designated 'S') is not present in serum-free broth; it is soluble in serum. It is not sensitive to oxygen, but is very susceptible to heat, even at 55° C.; it also deteriorates at room temperature. It is non-antigenic in the form in which it is extracted by serum from the streptococcal cells, but immunisation of animals with the living organisms gives rise to S (as well as O) anti-hæmolysin. As judged by neutralisation reactions with antisera, the two hæmolysins are separate antigens.

Among other diffusible products a leucocidin and a fibrinolysin have been demonstrated. Some evidence has been brought forward to show that leucocidin production can be correlated with virulence. Tillett and Garner have found that broth cultures and also filtrates of *S. pyogenes* lyse human fibrin within forty-five minutes and that serum from a patient convalescent after infection by this organism inhibits the fibrinolytic action. The fibrinolytic activity is destroyed by heating at 100° C. for sixty minutes. It is proportional to its fibrinolytic

activity (Tillett). The scarlatinal toxin produced by strains of *S. pyogenes* will be dealt with later. An exotoxin capable of causing an immediate lethal effect on animals after intravenous injection has also been described (Harris). In addition to these exotoxins, substances of the nature of endotoxin have been demonstrated in streptococcal cultures. These will be discussed in relation to scarlatina and acute rheumatism.

Reference has already been made to hyaluronidase as a product of *S. pyogenes* and its relationship to capsulation of the organism. It is now recognised that this agent is capable of increasing tissue permeability and

may play a part in the rapid spread of the streptococcus in the tissues. Crowley, however, has pointed out that it is produced by only a limited number of strains and he considers it is not related to virulence. The production of hyaluronic acid and hyaluronidase by *S. pyogenes*, and their relations are problems which require further

S. pyogenes, though the prevalent form of streptococcus in the commoner pyogenic infections, constitutes only one species of a large biological group, and comprises in itself a number of types differing in serological characters. The streptococcus group is thus heterogeneous, and apart from the typically pathogenic forms embraces species or types which are common commensal organisms flourishing in the mouth, throat, intestines, etc. Moreover, streptococci derived from animals often present features that differentiate them from those usually found in the human subject. The biological classification of all these organisms presents many difficulties, and different systems of classification and nomenclature have been used at different times. Morphology, biochemical properties, hæmolytic action, and other visible effects on blood incorporated in culture medium, resistance to heat, habitat (among the commensal types), relationship to disease, aerobic or anaerobic characters, serological reactions, etc., have all been utilised in classification and nomenclature. Failure to form catalase is a characteristic of the whole group.

MORPHOLOGY. *S. pyogenes* shows chains of moderate length as a rule; commensal streptococci of the mouth and throat vary in this respect, and long-chained forms are sometimes met with. The faecal streptococcus (generally designated 'enterococcus') occurs usually either as diplococci or short chains. The shape of the individual cocci varies; *S. pyogenes* consists usually of spheroidal forms, but among the mouth and throat organisms the cocci may be oval or elongated. The enterococcus consists of relatively large oval cocci, which may be lanceolate in appearance and resemble the pneumococcus. Certain varieties are markedly capsulate and the differentiation of these from the pneumococcus has sometimes presented difficulty. For all practical purposes, however, morphological differences are of little significance in the biological classification of these organisms.

HÆMOLYSIS AND OTHER VISIBLE CHANGES PRODUCED IN BLOOD MEDIA. The changes produced by streptococci when growing on a medium containing blood have provided most valuable criteria for the recognition of certain main groups of these organisms. Schottmüller first employed the appearance of the colonies of streptococci on blood agar as a means of separating varieties. The medium consisted of 2 parts human blood (rabbit, horse, or ox blood may likewise be used) and 5 parts melted agar; it is, however, better to add the blood in the proportion of 5 to 10 per cent. He distinguished the '*Streptococcus erysipielatis*' (corresponding to *S. pyogenes*), which formed grey colonies and had a marked hæmolytic action, the '*Streptococcus viridans*', which produced small colonies of green colour, ascribed to formation of methæmoglobin, and very little hæmolysis. Mandelbaum added to these the '*Streptococcus saprophyticus*', which was without hæmolytic action. Smith and Brown studying the appearances of cultures on horse blood agar, designated the hæmolytic streptococci as type β , the *viridans* strains as type α . They regarded the α type as weakly hæmolytic and drew attention to the partial hæmolysis in the discoloured zone round colonies on blood agar. The commensal streptococci of the mouth and throat are mostly of the α or *viridans* type. A further type of reaction on blood medium was described and designated α' hæmolysis; in this case there is an area of lysis round the

colonies, but it is somewhat less clearly defined than the typical β hæmolysis and discoloration is absent. The 'Streptococcus saprophyticus' of Mandelbaum is Smith and Brown's type; γ it is entirely devoid of hæmolytic properties and causes no discoloration on a blood medium. The enterococcus is of this type.

results of this test for hæmolysis and those obtained by adding fluid culture to a blood suspension. Thus certain streptococci may be of the β or hæmolytic type on blood agar, but produce no hæmolysin demonstrable in fluid culture. It has been generally supposed that the green coloration of *S. viridans* on blood agar is due to methæmoglobin. The colour change, however, is more marked when the organism is growing in a medium containing heated blood, being yellow-green; it is favoured by the presence of acid. The yellow colour of growths of *S. viridans* and the pneumococcus on heated-blood media is proportional to the amount of hydrogen peroxide which they produce, and this colour change is somewhat similar to that produced artificially.

The green coloration of heat product of hæmatin. Green coloration, however, is not produced by the action of peroxide alone, either on heated or unheated blood, and the nature of this colour effect of *S. viridans* is still obscure.

It is doubtful, whether the distinction between hæmolytic and non-hæmolytic streptococci, as judged by appearances on blood agar, is fundamental, since there is evidence that in the animal body the non-hæmolytic type may be derived from the hæmolytic. Thus, it has been found after inoculation with a culture of hæmolytic streptococci that a proportion of the organisms recovered a few hours later are non-hæmolytic and reduced in virulence. Fry (1933) has shown that streptococci, derived directly from the body, when grown aerobically may sometimes appear to be of the *viridans* type, and yet under anaerobic conditions yield colonies with marked rings of hæmolysis, and without any green coloration, and when

hæmolytic variants to produce typical β hæmolytic colonies. Growth under increased oxygen tension leads to the development of *viridans* variants (Isaacs).

colc
of s
are of the hæmolytic type (β), the usual mouth and throat commensals of the *viridans* (α) type, and the enterococcus of the γ type.

S. pyogenes, as has been indicated above, is the classical species among the hæmolytic streptococci and that most frequently present in human disease, but it has been long recognised that these hæmolytic organisms

named '*S. salivarius*', streptococci of different origin and habitat, e.g. *S. bovis* and *S. equinus*, exhibit the same green coloration on blood agar.

Much work has been done by D. Thomson and by Warren Crowe with a view to subdividing and classifying the non-hæmolytic streptococci, mainly by appearances of colonies on inspissated or coagulated blood media. Thomson has used a medium of

fæces, vagina, and urine. He points out that an enormous number of different varieties of these streptococci can be recognised in this way, and that the mouth and upper respiratory passages constitute their principal reservoir. He has shown that the streptococcal flora of the mouth is in a state of constant variation and also varies widely among different individuals, thus, on any one occasion many different varieties may be present in the saliva. He finds that the streptococcal flora of animals differs considerably from that of man. He finds that certain types may be common to both. Thomson has described a group which is associated with other bacteria.

The main object of these studies has been to determine whether particular types are specifically associated with pathological conditions, and one variety has been found related to the common cold. For details the original papers should be consulted.

BIOCHEMICAL PROPERTIES AND OTHER CHARACTERS. *Fermentation re-*

growth and when passed through the animal body (Ainley Walker; Beattie and Yates). Gordon, and Andrewes and Horder originally employed a series of biochemical reactions for the differentiation of types (clotting of milk, reduction of neutral red, acid fermentation of lactose, saccharose, raffinose, salicin, coniferin, and mannitol); and six varieties, of which five occurred in the human subject, were recognised in this way: (1) *S. mitis* occurring normally in the saliva and fæces, (2) *S. pyogenes* the common pathogenic type, (3) *S. salivarius* occurring normally in the mouth, (4) *S. anginosus* found in inflammatory conditions of the throat, (5) *S. faecalis* occurring normally in the fæces and corresponding to the organism now designated enterococcus, (6) *S. equinus* which was found to be common in the air and dust of towns and was derived apparently from horse dung. This system of classification is now only of historical interest. Or have frequently been stressed as ch raffinose fermentation of salivary st mentation of the fæcal streptococci.

tion of hæmolytic streptococci from human and animal sources respectively can often be made by fermentation tests with sorbitol and trehalose, human strains fermenting trehalose but not sorbitol, animal strains fermenting sorbitol but not trehalose (Edwards). The final acidity produced after growth in 1 per cent. glucose broth is a further distinguishing feature, the pH with the prevalent streptococcus of bovine mastitis (*S. agalactiæ*) being not over 4.8, while with *S. pyogenes* the pH is over 5. The enterococcus yields a final pH of 4.0 to 4.2.

Certain other reactions have also been employed for differentiating members of the group. Hydrolysis of sodium hippurate is effected by *S. agalactiæ*, the organism usually met with in bovine mastitis. Ability to produce growth on media containing bile salts, e.g. MacConkey's, is a feature of the enterococcus, the usual pyogenic streptococci and streptococci from the mouth and throat usually fail to grow in the presence of bile salts. Resistance to heat is a criterion in the separation of the enterococcus from other varieties (*vide infra*). The temperature range of growth may also be a characteristic, e.g.

S. lactis (the saprophytic streptococcus associated with the curdling of milk) and the enterococcus grow at 10° C., whereas *S. pyogenes* and the mouth and throat commensals are unable to flourish at this temperature. The enterococcus also differs from *S. pyogenes* in its ability to grow at 45° C. Growth in the presence of 6.5 per cent NaCl and growth at a pH of 9.6 characterise the enterococcus. Reducing properties are strongly shown by *S. lactis* and most strains of enterococcus, in contrast to other streptococci. Certain streptococci when first isolated exhibit anaerobic or micro-aerophilic characters and some of these have a putrefactive action in cultures.

Enterococcus. This designation has been applied to the common type of intestinal streptococcus to which the name *S. faecalis* was originally attached. When growing in the body it usually occurs as a diplococcus, the individual organisms being oval or lanceolate like the pneumococcus, the members of a pair are often set at an angle and unequal in size. In cultures it shows considerable pleomorphism, and tends to grow in masses, though short chains occur in fluid media (Fig. 12). On the surface of agar it produces a thin, semi-transparent layer with smooth margins, and there is not the tendency to form separate colonies which is shown by most streptococci. Isolated colonies are usually somewhat larger than those of other streptococci. It produces a diffuse turbidity in broth, with the formation after a time of a somewhat glairy deposit, sometimes there is a scum on the surface. It flourishes well at a lower temperature (e.g. 20° C.) than that at which *S. pyogenes* will grow, and has great longevity in cultures. Growth occurs at 10° C. to 45° C., at a pH of 9.6, and in the presence of 6.5 per cent NaCl. On blood agar there is no hæmolytic and no green coloration. This organism is representative of Smith and Brown's γ type (*vide supra*). Some streptococci, however, which correspond in other characters to the enterococcus may be hæmolytic on blood agar, e.g. varieties designated *S. zymogenes* and *S. durans*. It possesses a relatively high resistance to heat: broth cultures survive exposure to 60° C. for thirty minutes, while other streptococci are killed in five to ten minutes at this temperature. In testing for heat-resistance the reaction of the culture fluid should be neutral. When first isolated, some strains have been found to prefer anaerobic conditions. It ferments lactose, mannitol, salicin, and æsculin, and coagulates milk. It differs from other types of streptococci in its ability to grow on a medium containing bile salt. Hydrogen sulphide is produced in cultures. Some strains liquefy gelatin. Most strains have strong reducing properties in culture.



FIG. 12. Enterococcus, from a twenty-four hours' culture on MacConkey's medium. Stained Gram's method $\times 1,000$.

Anaerobic Streptococci. Organisms of this group were first described by Veillon. In morphology they are typical Gram-positive streptococci, though occurring frequently as diplococci or in short chains. In many strains the cocci are exceptionally small (about 0.4 μ). They require an atmosphere containing excess of carbonic acid and appear either as strict anaerobes or

micro-aerophiles when first isolated, and grow best at body temperature. A suitable medium is one containing glucose and blood or serum. Some strains produce gas and putrefactive odour in culture medium. They show varied fermentation reactions like the aerobic types; hæmolytic action is not usually present on blood agar, but hæmolytic strains have been included in the group. Sometimes colonies on blood agar show a black discoloration. They have been found normally on various mucous membranes, and have been isolated from gangrenous conditions of the urogenital tract, intestine, and lungs, and in various septic lesions. They occur also in puerperal sepsis and in this condition they may produce a general infection. Recently attention has been drawn to a massive infection of muscles by anaerobic streptococci along with *S. pyogenes* and staphylococci, following infected wounds and closely similar to gas-gangrene due to the usual anaerobic bacilli. The diagnosis of such streptococcal myositis may be made by microscopic examination of stained smears from the affected muscles. Hæmolytic strains have been found in a characteristic spreading ulcerative condition of the skin. The classification of these anaerobic streptococci requires further investigation.

Aronson's Streptococcus. This type is of special interest in virtue of its resemblance in certain respects to the pneumococcus (*q.v.*). As pointed out by Griffith (1935) it is similar to this organism in its colony structure, virulence for mice and rabbits, the formation of capsules, the production of specific precipitable substance, and the occurrence of transformation to the R form associated with attenuation in virulence. On the other hand, it differs from the pneumococcus in the spherical shape of the cocci, the absence of autolysis in surface colonies, insolubility in bile, the production of a hæmolysin (like that of the classical *S. pyogenes*) and the β hæmolysis of deep colonies in blood agar. This hæmolysin, however, is intimately associated with the organisms and is practically removed from a fluid culture by centrifuging out the streptococci (Howie). Griffith recorded its isolation from the throat of the human subject, but did not regard it as pathogenic. In Lancefield's serological classification (*vide infra*) it apparently falls into the group which comprises the streptococci of bovine origin.

Streptococcus mucosus. Non-hæmolytic streptococci producing thick easily demonstrable capsules and yielding mucoid growths on culture medium have been described under this name. Their insolubility in bile and the absence of inulin fermentation distinguishes them from the pneumococcus, although, like Aronson's streptococcus, they may constitute intermediate forms. Capsulate hæmolytic streptococci producing mucoid growths have been described in milk-borne epidemics of sore throat and designated *S. epidemicus*, but it is doubtful if these merit separate mention; the formation of mucoid colonies, as already shown, may be a variant character of *S. pyogenes*.

Streptococcus agalactæ. This designation has been given to strains of streptococci found in the subacute and chronic forms of bovine mastitis and presenting the following characters: chains long; growth in broth flocculent, milk acidified and coagulated in forty-eight hours; strains vary as regards appearance of colonies on blood agar and include α , β , and γ types; the β strains produce a filterable hæmolysin which is oxygen-stable; sodium hippurate is hydrolysed, glucose is fermented, also saccharose and trehalose, but not mannitol and sorbitol, strains vary as regards fermentation of lactose and salicin; final pH of growths in glucose broth 4.2 to 4.8; killed at 60° C (thirty minutes); does not reduce methylene blue in milk. The effect on sodium hippurate is tested by growing the streptococcus in broth

containing 1 per cent. of this substance for five days at 37° C., then 0.2 to 0.4 c.c. 12 per cent. ferric chloride solution containing 2 per cent. concentrated hydrochloric acid is added to 1 c.c. of the culture. A precipitate remaining insoluble indicates the presence of benzoate, which denotes hydrolysis of the hippurate. As will be shown later, *S. agalactiae* corresponds to Lancefield's serological Group B.

Another designation given to this organism is Group I of the streptococci associated with mastitis and two other groups of these organisms have been recognised. Group II ('*S. dysgalactiae*') is associated with the acute or sub-acute disease. The organisms are non-haemolytic, reduce methylene blue in milk, do not ferment mannitol, salicin, or inulin. Group III ('*S. uberis*') are also non-haemolytic, acidify milk at 10° C., reduce methylene blue and ferment mannitol, salicin, and inulin. It should also be noted that haemolytic streptococci may occur in bovine mastitis, differing from *S. pyogenes* in fermenting sorbitol but not trehalose. These can be assigned to Lancefield's serological Group C.

Streptococcus lactis, the streptococcus found in milk and responsible for curdling, has generally been regarded as similar to the enterococcus, but it has been shown recently by Shattock and Mattick that it differs clearly from the latter in its failure to grow at 45° C. or at pH 9.6, and its sensitivity to heating at 60° C. for thirty minutes under the conditions of their experiments. While saccharose is usually fermented by the enterococcus, *S. lactis* does not ferment this sugar. The serological difference of these two organisms is dealt with later.

Differential Characters of Streptococci commonly met with in the Human Subject :

A. AEROBIC

(1) 'Haemolytic' Streptococci, *producing filterable haemolysin*. Chains of moderate length consisting of spheroidal cocci, viability in culture moderate, salicin usually fermented, mannitol usually not fermented, killed at 60° C. within thirty minutes, growth inhibited by bile salt. The great majority of these organisms are *S. pyogenes*.

(2) 'Non-haemolytic' Streptococci, *filterable haemolysin not demonstrable in cultures*.

(a) *Mouth Streptococci*. Chains frequently long and cocci elongated; viability in culture weak, peroxide production well developed; on blood medium produce green coloration (*S. viridans*), though some strains show no visible alteration of blood, frequently ferment raffinose, mannitol not fermented, killed at 60° C. within thirty minutes, growth inhibited by bile salt.

(b) *Faecal Streptococci (Enterococcus)*. Cocci oval and in pairs or short chains, viability in culture considerable, peroxide not formed, on blood medium no visible alteration in colour, usually ferment mannitol and aesculin; resist 60° C. for thirty minutes; growth not inhibited by bile salt, produce H₂S, some liquefy gelatin.

B. ANAEROBIC. Distinguished by their anaerobic or micro-aerophilic characters, usually non-haemolytic.

Serological Classification of Streptococci. Attempts have been made to classify streptococci by serological methods—agglutination and precipitation reactions with specific antisera. Special attention has been devoted to the haemolytic streptococci. Serologically these streptococci represent a heterogeneous group. Earlier workers on this subject attempted to classify them by agglutination reactions into well-defined types and to differentiate the scarlatinal strains from the ordinary pyogenic streptococci in this way.

This system of classification, as has been shown, though originally applied to the hæmolytic streptococci also includes organisms of the non-hæmolytic category. In connection with the practical application of grouping tests for tracing the origin of streptococci, it should be noted that rapid passage through mice of a Group A strain has been found to lead to loss of the group carbohydrate without the acquisition of carbohydrate of another group (Wilson)

Method of determining the serological group Precipitating sera for the various groups are obtained by immunising rabbits with representative strains. Cultures in horse-flesh infusion are made in 10 c.c. of broth, 1 per cent. of formalin is added, and the mixture is allowed to stand for 24 hours. The mixture is then centrifuged and the supernatant fluid pipetted off and mixed with 0.5 c.c. of acetone. The mixture is centrifuged and the supernatant discarded. 1 c.c. of normal saline is added and a drop of phenol red indicator, and the solution is neutralised with solid sodium carbonate. This extract is then used for the test. The precipitation reaction is determined by adding varying concentrations of the extract (undiluted, 1 in 3 and 1 in 9) to an equal volume of undiluted serum in narrow tubes, the extract being carefully superimposed on the serum. Precipitation occurs at the interface within five minutes and is easily observed. To economise serum the test may be carried out in capillary tubes.

Griffith's types These have been designated numerically 1, 2, 3, etc. As already mentioned, most of them belong to Group A, and the most frequent are types 1, 2, 3, 4, 6, and 8. The types which do not fall into Group A are 7, 16, 20, and 21¹.

Antisera are obtained by injecting rabbits with repeated doses of killed cultures of representative strains. Such antisera show many cross-reactions, but these effects can be partially or wholly removed by absorption; and for final routine testing absorbed sera as free as possible from cross-reactions are used. In determining the type of an unknown strain, a preliminary agglutination test is made with pooled sera, each 'pool' representing four or five types. After ascertaining with which pooled serum the organism reacts, the actual type can be determined with the individual sera comprising the pool.

For the test the streptococci are grown in rabbit serum broth for eighteen to twenty-four hours. The supernatant fluid after centrifuging is removed and a dense suspension made by emulsifying the sediment in distilled water. By uniform growth. The possibility of this procedure the original antigenic characters of the strain may be modified or lost. The actual test is usually performed with undiluted serum by the slide method, and clumping is visible to the naked eye or with a low magnification of the microscope within a few minutes.

There is evidence that other groups besides A can be subdivided by agglutination reactions into multiple serological types. Thus, three main types have been recognised in Group B (Stableforth).

The *viridans* streptococci have likewise been shown to be serologically heterogeneous, but so far such serological studies have not led to any definite classification.

Practical applications of serological classification. Lancefield's grouping of the hæmolytic streptococci has undoubtedly proved of value in the identification of strains which are of human origin and potentially pathogenic to man—Group A, but though most of the strains isolated from human disease can be assigned to Group A, members of other groups, as has been noted, may occur either as commensals or in actual infections, some even of

¹ 7, 20, and 21 belong to Group C; 16 to Group G.

a severe nature. Group B is of special importance in veterinary medicine as it comprises the strains most frequently associated with bovine mastitis, though strains of other groups may occur in this disease. Group C is also of veterinary importance. The outline given above of the various groups illustrates the practical relations of this system of classification.

medical or nursing attendant, it has been generally assumed that the infection has been derived from the latter. If the same type is found in several cases which have some association with one another, this would indicate a common source of infection or the spread of the infection from one case to the other. If in hospital in a case of scarlet fever presenting some suppurative complication, the strain isolated from the latter differs in type from that present in the primary infection, it may be concluded that the complication is due to a new infection.

The practical applications of serological classification will be illustrated further in the discussion of various forms of disease produced by the hæmolytic streptococci.

Experimental Inoculation. *S. pyogenes* is an organism the virulence of which varies much according to the diseased condition from which it has been obtained, and also one which loses its virulence rapidly in cultures. Even highly virulent cultures, if grown under ordinary conditions, in the course of time lose practically all pathogenic power. By passage from animal to animal, however, the virulence may be much increased, and *pari passu* the effects of inoculation are correspondingly varied. Marmorek, for example, found that the virulence of a streptococcus could be enormously increased by growing it alternately (a) in a mixture of human blood serum and broth, and (b) in the body of a rabbit, ultimately, after several passages it possessed a super-virulent character, so that even an extremely minute dose introduced into the tissues of a rabbit produced acute septicæmia, with death in a few hours. It has been proved that the same streptococcus may cause at one time merely a passing local hyperæmia, at another a local suppuration, at another a spreading erysipelatous condition, or again a general septicæmic infection, according as its virulence is artificially increased. Such experiments are of extreme importance as explaining to some extent the great diversity of lesions in the human subject with which streptococci are associated. In addition to the general infection produced by virulent strains, localisation may occur in certain tissues, e.g. joints and muscles, with associated lesions, while hæmolysis and anæmia may result from the action of the toxins. The leucocidin enables the organism to break down the first defences of the body and the fibrinolysin and spreading factor, if produced, may facilitate spread

highly virulent for mice

Non-hæmolytic streptococci. In animal experiments these streptococci are usually of low virulence. Large doses of culture can often be inoculated without obvious pathogenic effect. After intravenous injection they may sometimes localise in the endocardium, producing endocarditis with vegetations, the lesion being somewhat similar to that of subacute bacterial endocarditis of the human subject. Some workers have found also a selective localisation in synovial membranes, the pericardium, kidney, and in the cerebro-spinal fluid. Such results have suggested the relation of organisms

of this type to acute rheumatism and its complications, but recent evidence has indicated that hæmolytic strains are associated with this disease (*vide infra*). Localisation in the gall-bladder with resulting cholecystitis and calculus formation has also been recorded in the case of streptococci isolated from the gall-bladder and cystic lymph gland of cases of cholecystitis in the human subject; but streptococci from other sources may localise similarly in the gall-bladder and produce cholecystitis.

Lesions in the Human Subject. *Hæmolytic streptococci* are especially found in spreading inflammation with or without suppuration, in diffuse phlegmonous and erysipelatous conditions (*vide infra*), suppuration in serous membranes and in joints. They are common in wound suppurations, usually along with other pyogenic organisms. During the war of 1914-18 streptococci were usually to be found in gun-shot wounds; at first the enterococcus abounded, at a later stage hæmolytic streptococci were the more common type. In most cases this was the result of hospital infection. These organisms may also cause acute suppurative osteomyelitis and ulcerative endocarditis (*vide infra*). Lymphangitis and secondary abscesses in lymphatic glands are frequently caused by them. These lesions often occur very quickly when a virulent streptococcus has gained entrance through a prick or scratch in the skin, and in such cases there may be little or no inflammatory reaction at the site of entry of the infection. Impetigo can be generally attributed to hæmolytic streptococci. They produce fibrinous exudation on mucous surfaces, leading to the formation of false membrane, e.g. in the throat in scarlatina and other conditions, and they are also the organisms most frequently present in acute catarrhal inflammations in this situation. The relation of streptococci to scarlatina will be discussed in detail later. Epidemics of sore throat, often of a severe type, have been attributed to streptococci spread by milk and derived from udder infection in the cow. Suppurative otitis media with its sequelæ—e.g. mastoiditis, meningitis—is frequently streptococcal in origin. Hæmolytic streptococci may be found in the broncho-pneumonic conditions occurring as complications of other infections, e.g. influenza. In puerperal sepsis they are frequently found in pure or mixed infection, and they appear to be the most frequent cause of puerperal septicæmia (*vide infra*). In pyæmia they are frequently present, in some cases associated with other pyogenic organisms. The question of their relationship to acute rheumatism will be referred to later. It has been reported by Jordan and Burrows that poisoning may result from the ingestion of food contaminated by streptococci, just as in the case of staphylococci. The epidemiological aspects of streptococcal infections are dealt with later.

Non-hæmolytic streptococci. These are generally of lower virulence and associated with less acute infections. They are slowly invasive, and suppuration and tissue destruction are not marked features of the inflammatory lesion. They are frequent in tonsillitis, otitis, dental abscess, pyorrhœa alveolaris, and bronchitis. They may occur, however, in broncho-pneumonia and even in septicæmic conditions. Non-hæmolytic streptococci, both of the *viridans* and enterococcus types, are associated with a certain proportion of cases of cholecystitis and can be isolated from the bile, gall-bladder wall, or cystic lymph gland. The condition of subacute bacterial endocarditis (*vide infra*) is usually due to the *viridans* type. Some cases of enteritis in infants have been attributed to the enterococcus. In late cases of bacillary dysentery this organism may be present in the stools in very large numbers suggestive of a secondary infection. The lesions in which the enterococcus is found are mainly those where infection can be traced from the bowel, e.g.

cystitis, appendicitis, peritonitis, cholecystitis. It has been found also in abscesses following typhoid fever, in middle ear disease of infants, and in some cases of subacute endocarditis with metastatic suppuration in internal organs. It is difficult, however, to assess the pathogenicity of this organism in various conditions in which it may be found in a mixed infection.

pneumonia (of the horse), omphalophlebitis of new-born animals with metastases in liver and joints, and suppurative cellulitis. Reference to the serological groups responsible for animal disease is made on p. 161. Bovine mastitis is one of the most important and prevalent of animal diseases due to streptococci. The condition is generally regarded as transmissible among the animals of dairy herds, the organisms being conveyed from the udder, teats, and milk of an infected animal to the udder and teats of others by the hands of milkers, the cups of milking machines, etc. The commonest form of streptococcus in subacute and chronic mastitis is the *S. agalactiae* (Lancefield's Group B). Two other types of streptococci associated with mastitis have been recognised (p. 159), and hæmolytic Group C strains have also been found. It has proved difficult to establish the causal relations of *S. agalactiae* to mastitis by experimental inoculation of the udder, and though this organism and the other streptococci referred to have an undoubted association with the disease and are probably the exciting agents of the active lesions, other predisposing factors appear to play an etiological part, these are still undefined. The question, of course, has arisen whether such organisms are the primary infecting agents or whether their rôle is secondary, this also requires further investigation. It should be noted that other organisms besides streptococci may be associated with bovine mastitis, e.g. staphylococci and *Bacillus* (or *Corynebacterium*) *pyogenes*. *S. pyogenes* (Group A) may sometimes be associated with an acute form of mastitis, but in this case the infection is derived from a human source among those handling the animals; in turn, the milk from the infected animals may spread the infection in the human subject, producing, for example, a severe form of epidemic sore throat.

Scarlatina. It has been long recognised that streptococci are usually present in large numbers in the throat in scarlatina, and that many of the complications of this disease are undoubtedly streptococcal. At one time streptococci were regarded, in virtue of their occurrence in the disease, as the likely etiological agent, and the associated strains were designated *S. scarlatinae*. Thus, in 1887 Klein as a result of the investigation of milk-borne outbreaks, claimed that the disease was due to a definite type of streptococcus, but subsequent work showed that the scarlatina streptococcus was similar in its cultural characters to the pyogenic strains and had no specific pathogenic effects on animals. Later views tended to reverse the original idea, although the almost constant occurrence of hæmolytic streptococci in the throat was well recognised, and it was supposed that these organisms represented a secondary undetermined factor. The filterability of the streptococci, however, led to negative results.

In 1923, G. F. and G. H. Dick were able to produce scarlet fever in the human subject by infecting the throat with a culture of a hæmolytic streptococcus isolated from a known case of the disease (a finding which has been

confirmed by subsequent workers); then they demonstrated that the scarlatina streptococcus produces a diffusible toxin, present in filtrates from cultures, which, injected intracutaneously in persons susceptible to the disease, elicits a reaction of cutaneous erythema and inflammation; while non-susceptibles (e.g. convalescents) generally fail to show the reaction. This reaction is therefore analogous to the Schick reaction for susceptibility to diphtheria (*q v*), and is designated the Dick reaction. They also produced symptoms of scarlet fever by injecting subcutaneously the filtrate of a culture of the scarlatina streptococcus. The validity of the Dick reaction has been generally accepted. Thus persons during the early stage of the disease (first three days), in the majority of cases, exhibit a positive reaction illustrating apparently their susceptibility to the toxin and the absence of any natural or acquired immunity. On the other hand, convalescents usually show a negative reaction, which suggests the acquisition of an antitoxic immunity as a result of the infection. Discrepancies, however, are sometimes observed between the results of the test and the clinical state (*vide infra*). It has also been shown by the so-called Schultz-Charlton reaction that the serum of convalescents contains a neutralising antitoxin, i.e. the intracutaneous injection of the convalescent serum (e.g. 0.2 c.c. of a 1 in 10 dilution) produces a local blanching or 'extinction' of the rash in an active case. Further, the serum of convalescents neutralises the Dick toxin when mixed with it and injected into the skin of a known susceptible person. These facts, together with the constant occurrence of hæmolytic streptococci in the throat, strongly support the view that scarlatina is due to infection of the throat with a hæmolytic streptococcus capable of producing a specific diffusible toxin which is responsible for the general manifestations of the disease, and that the infection is in the first instance local, the general condition being essentially a toxæmia. But immunity to scarlatina is dependent not only on antitoxin but also on antibacterial resistance, for it has been shown that infection by a scarlatina streptococcus does not necessarily produce scarlatina in a Dick-positive reactor (Schwentker *et al.*). The degree of toxigenicity of hæmolytic streptococci is also a factor in the production of scarlatina; thus, as shown by Hamburger, there may be seasonal variation in the capacity of these organisms to produce the characteristic syndrome.

As mentioned earlier, the question arose whether the streptococcus of scarlatina constitutes a specific type. This led to extensive studies of the serological characters of strains of hæmolytic streptococci from scarlatina and other conditions. The results at first suggested that the scarlatina streptococci might be classified in certain serological groups, but later observations showed that there is no line of demarcation serologically between the scarlatina strains and other hæmolytic streptococci. The serology of the hæmolytic streptococci has already been discussed and it has been found that strains from different types of lesion may belong to the same serological type. It has also been shown that hæmolytic streptococci from cases of erysipelas, infected wounds, and normal throats, may produce toxin similar to that obtained from known scarlet fever strains.

Practically all scarlatina streptococci can be assigned by precipitation reactions to Lancefield's A group, though occasionally strains belonging to other groups are found in the disease. By agglutination reactions the strains are distributed among the various types defined by Griffith, who stated, however, that a majority (over 60 per cent.) fall into four serological types, Nos 1, 2, 3, and 4. Though these four types are frequently represented among scarlatina strains they do not invariably predominate and in particular outbreaks a majority of the strains may belong to other types.

An obstacle to the experimental study of the etiology of the disease has been the fact that laboratory animals are generally insusceptible to the toxin. The only certain criterion, therefore, of the identity of a scarlatina-producing streptococcus depends on whether culture-filtrates in suitable dilution yield a positive Dick reaction in known susceptibles (*e.g.* early scarlet fever cases) and negative reactions in known negative reactors (*e.g.* convalescents). The neutralisation by an antitoxic serum (*vide infra*) of the reacting property of the filtrate constitutes a confirmatory test.

Though animals are usually insusceptible to the toxin even in large doses, it has been stated that young goats give a cutaneous reaction analogous to the Dick reaction (Kirkbride and Wheeler) and the same has been noted among chinchilla rabbits (Fraser and Plummer). Trask has also found that some rabbits exhibit susceptibility to the scarlatinal toxin, which is neutralisable by the specific antitoxin, and that susceptibility tends to increase with the age of the animal.

The logical outcome of research work on scarlet fever has been (1) the active immunisation with toxin for the prophylaxis of the disease, as in active immunisation against diphtheria (*q.v.*), and (2) the treatment of the disease by passive immunisation with antitoxic sera. By immunisation of horses with the toxin, a neutralising antiserum can be obtained, and such sera are utilised successfully in treatment.

The Dick reaction has confirmed that the age of maximum susceptibility to scarlet fever is between six months and five years. About 80 per cent of persons over twenty years of age are immune. This has been attributed to infection or subinfection in early life and the associated immunisation, but physiological maturity must also be a factor.

While the diffusible principle of the scarlatina streptococci, which is often spoken of as 'erythroxin', has been commonly regarded as analogous to other bacterial exotoxins, it has been suggested that the scarlet fever rash may be a phenomenon of supersensitiveness to the proteins of the streptococcus, and that the Dick reaction is an index of such sensitiveness (*vide*

reaction. This reaction, however, is not strictly analogous to the Dick reaction in the human subject (Mackie and McLachlan).

It has been shown that culture-filtrates as ordinarily used contain two reacting substances: a true exotoxin which is labile at 80° C. and is neutralised by scarlatinal antitoxin, and a nucleo-protein which is stable up to 100° C., though partially inactivated after half an hour at this temperature (Ando, Kurauchi and Nishimura). The usual Dick reaction is stated to be due to the presence of both substances, the reaction to the protein being allergic in nature. The latter may also be of the nature of an endotoxin. It has been customary in carrying out the control test for the Dick reaction to use a preparation heated to 100° C. for at least one hour in view of the difficulty of inactivating the material. This difficulty may be due to the reactive nucleo-protein referred to above. It has also been noted that during the course of the scarlatinal illness patients may develop a reactivity (on cutaneous testing) to the dissolved intracellular products of hæmolytic streptococci, indicating the acquisition of allergy at a time when they are becoming immune to the streptococcal exotoxin (Gibson and McGibbon). These observations serve to explain confusing results obtained at times in the application of the reaction, and a purified exotoxin free from bacterial protein has been advocated for the test.

abscesses (Figs. 14, 15). In some instances coliform bacilli have been found, and occasionally in endocarditis following typhoid fever the presence of the

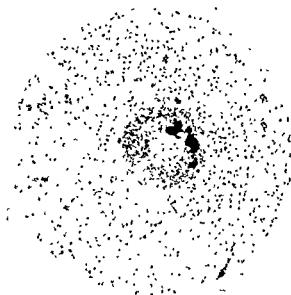


FIG. 11 Minute focus of commencing suppuration in brain—case of acute ulcerative endocarditis. In the centre a small hæmorrhage, to right side dark masses of staphylococci; zone of leucocytes at periphery. Stained by carmalum and Gram's method $\times 50$

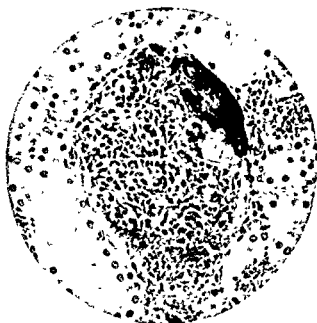


FIG. 15 Secondary infection of glomerulus of a kidney by

Stained by carmalum and Gram's method. $\times 250$

typhoid bacillus has been described. The meningococcus and the gonococcus have been reported in endocarditis, but such occurrences are relatively rare. Hæmophilic bacteria related to the influenza bacillus are also sometimes found. Tubercle nodules in the valves have been observed in a few cases of tuberculosis, though no ulcerative condition is usually produced.

Occasionally ulcerative endocarditis can be produced experimentally by the simple procedure of injecting staphylococci or streptococci into the circulation. It often follows, however, when the valves have been injured previously. Orth and Wyssokowitsch at a comparatively early date produced the condition by damaging the aortic cusps with a glass rod introduced through the carotid, and afterwards injecting staphylococci into the circulation. Similar experiments have since been repeated with streptococci, pneumococci, and other organisms, with like result. Rubbert found that if a potato culture of *Staph. aureus* was emulsified in salt solution, and then injected into the circulation, some minute fragments became arrested at the attachment of the chordæ tendineæ and produced an ulcerative endocarditis.

Focal Infection. This term is commonly applied in clinical medicine and surgery to chronic localised inflammatory or suppurative lesions from which bacteria may enter the blood stream and then localise in some other part of the body, or from which bacterial products are absorbed and produce lesions in other parts either by direct toxic action or as a result of an allergic state. Such chronic focal infections are not uncommon in various situations, e.g. teeth, gums, tonsils, nasal sinuses, uterus, etc. Streptococci are frequently the associated organisms and a variety of conditions of doubtful etiology has been attributed to this cause, e.g. rheumatoid arthritis, chronic rheumatism, cholecystitis, pancreatitis, irido-cyclitis, gastric and duodenal ulcer. The evidence on this question is quite inconclusive though undoubtedly systemic effects and remote lesions may result from such chronic septic foci.

The Source of Streptococcal Infections. The mouth and throat are the natural habitat of certain non-hæmolytic streptococci and the question arises whether the hæmolytic streptococci can establish themselves as commensals in the same situation. They are certainly not regular inhabitants of the throat, but some observers have recorded a high prevalence, even in 61 per cent of healthy persons. They have been detected in 97 per cent of excised tonsils (Pilot and Davies), and it has been suggested that the tonsillar crypts habitually harbour these organisms. Other observers have considered that their presence is associated with, or follows, a local pathological condition. Remarkable fluctuations in prevalence in apparently healthy persons have been described, e.g. from zero to 36 per cent in the same group of persons studied over a period (see Topley and Wilson), though usually ranging from 5 to 20 per cent. A proportion of these hæmolytic strains found in the throats of healthy persons do not belong to Group A and their potential pathogenicity is doubtful. However, a 7 per cent average prevalence of Group A streptococci has been recorded in normal adults (Hare). We have no definite knowledge of all the factors that determine the frequency of these organisms in the throat, though some workers have found a low prevalence in tropical and subtropical regions as compared with temperate climates. A seasonal fluctuation has been described in New York by Coburn—the prevalence being lowest in late summer. It would appear that on the whole the prevalence is highest in the large communities of temperate regions during the cold season when catarrh of the respiratory passages is common and when people tend to be most closely aggregated in insufficiently ventilated places. Conditions of communal life in temperate climates seem most favourable to these organisms' commensal and parasitic relationship with the human body.

It has also been shown that epidemiologically each serological type is independent. A heightened prevalence of an infection by hæmolytic streptococci may be due to the increased incidence of only one type, but often more than one is operative and each causes, as it were, a separate epidemic running an independent course. Collective immunity to one type does not confer resistance to another and continued prevalence may be the sum of infection

Bacteriological Examination of Inflammatory and Suppurative Conditions. This includes (1) microscopic examination, (2) the making of cultures and, if necessary, subsequent biological, chemical, and serological tests with these.

(1) The pus or other fluids should be examined microscopically, first of all by means of film preparations in order to determine the characters of the organisms present. The films should be stained by Gram's method, and it may be of value to use also one of the ordinary staining solutions, such as a watery solution of methylene blue. The former is of course essential in the recognition of the pyogenic cocci.

(2) The cultivation and separation of the pyogenic cocci from the lesions are best attained by the method of successive strokes on blood agar plates. In this way discrete colonies are obtained and their various characters can be ascertained, including the microscopic appearances of the organisms in films stained by Gram's method, etc. Individual colonies can, if necessary, be subcultured, so that pure cultures are available for further study including biochemical and other special tests necessary for the complete identification of the particular organism and its variety, group, type, etc. Animal inoculation experiments may be carried out if required.

In cases of suspected septicæmia or pyæmia blood culture is carried out.

Reference is made in the Appendix to the methods of obtaining suitable specimens for bacteriological examination. The bacteriological examination of infected wounds in which there may be a considerable mixture of different organisms including anaerobes will be dealt with in Chapter XX.

CHAPTER V

THE PNEUMOCOCCUS (*DIPLOCOCCUS PNEUMONIÆ*)

INFLAMMATORY changes in the lungs are the results of different kinds of infection and lead to a variety of structural changes. Thus various forms—lobar pneumonia, broncho-pneumonia, hypostatic pneumonia, and embolic pneumonia—are recognised. Acute lobar pneumonia, however, stands out in certain respects from the others and presents characters of special interest. Its striking clinical features and the course of the fever, especially the crisis at the time of recovery, have long been recognised as resembling those of a specific infective disease. Further, its occurrence in epidemics supports this view.

Our knowledge of its etiology has been gradually evolved, rather than established by one discovery as has been the case with other infections, and the pneumococcus before it gained

This was due to several circumstances. The organism was found both in other lesions and in normal throats, and also to the difficulty experienced in producing a typical lobar pneumonia in animals. The causal relationship of the pneumococcus to acute lobar pneumonia was conclusively established by A. Fraenkel in 1886, though it had been described in this condition by earlier observers, notably Friedländer and Talamon. Confusion, however, arose as the result of the former's isolation of the capsulate bacillary organism now known as the pneumobacillus. The position was finally clarified by Fraenkel and by Weichselbaum, and the two organisms were clearly differentiated. It was also shown that the pneumobacillus is only infrequently present in the disease. The general result of all later observations on pneumonia has proved that the pneumococcus is the organism causally related to the vast majority of cases of acute lobar pneumonia.

MICROSCOPIC CHARACTERS As seen in pneumonic sputum or exudate, the pneumococcus occurs in the form of an oval coccus, about $1\ \mu$ in its longest diameter, arranged generally in pairs (diplococci), but also in chains of four to ten (Fig. 16). The free ends are often pointed like a lancet while the proximal poles are rounded, hence an earlier name '*Diplococcus lanceolatus*'. The long axes of the two cocci are in line. These cocci, in their typical form, have round them a continuous capsule, which in films stained by ordinary methods usually appears as an unstained zone, but is sometimes coloured more deeply than the ground of the preparation. The capsule is rather broader than the body of the coccus, and has a sharply defined external margin (Fig. 16), it may be differentially stained by special methods (*vide Appendix*) (Fig. 17) and is also revealed by negative or relief staining (Fig. 18). In sputum preparations the capsule of the pneumococcus may not be so easily

the tissues and in culture, the cocci in this form tend to be more spheroidal than other types. The capsule of the pneumococcus is demonstrable in recently isolated cultures in a medium enriched with blood or serum (*vide infra*), but may appear to be absent in ordinary media, and after continuous

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cultivation the power of capsulation is lost in association with attenuation or loss of virulence of the strain. In culture the morphology of the organism may deviate considerably from the typical characters observed when it is growing in the tissues; the cocci may be spherical rather than oval or lanceolate; and sometimes elongated forms are seen, even resembling bacillary organisms. Chained forms are also more frequent; and degenerate or involution forms are found in old cultures. The biological importance of

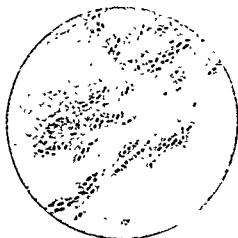
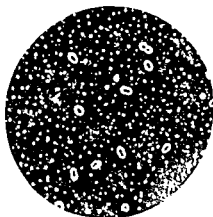


FIG. 16. Film pneumococcus.

FIG. 17. Pneumococcus in serous exudate at site of inoculation in a mouse. Stained to show capsule. $\times 1,000$ FIG. 18. Pneumococcus, film from lung exudate in lobar pneumonia. Eosin capsule method. $\times 1,000$

the organism may degenerate individuals may occur, and these have often become Gram-negative. The same variation is observed in culture.

CULTURAL CHARACTERS A pure culture can readily be obtained by injecting pneumonic sputum beneath the skin of a rabbit or a mouse: in about twenty-four to forty-eight hours the animal will die, with numerous capsulate cocci throughout its blood, from the heart-blood pure growths on culture medium can then be obtained. Pure cultures can also be got *post mortem* from the lungs of pneumonic patients by inoculating blood agar with

a scraping taken from the area of acute congestion or commencing red hepatisation. Cultures made from pneumonic sputum usually yield a mixed growth with varying numbers of the pneumococcus present.

The pneumococcus is an aerobe and facultative anaerobe, and its optimum temperature is about 37°C ., growth does not occur as a rule below 22°C . Strains in which the virulence has disappeared often grow well at 20°C . Growth is in general improved by an increase of carbon dioxide in the atmosphere. Strains of the pneumococcus have been described which in primary culture are obligate anaerobes or micro-aerophilic. It grows on ordinary media, provided these contain a meat infusion prepared from fresh muscle tissue (commercial meat extracts are not a suitable substitute), peptone, sugar (in small amount), and mineral salts, and are subjected to the minimum heating to dissolve the constituents and sterilise the

Wright
glucose,
peptone
affords a suitable supply of thermostable nitrogenous substances, while yeast extract, blood, meat extract, and serum contain the thermolabile constituents which are essential for growth. Heating of organic fluids used for

pointed out that one of the difficulties in producing a satisfactory medium is due to the incomplete reduction of the peptone, but that this is overcome by adding the peptone to the other constituents of the broth before heat is applied, thus exposing it to the reducing action of the minced meat during subsequent heating. The pH should be from 7.8 to 8.0. In making subcultures a relatively large inoculum should be used. While cultures can be obtained in this way on ordinary media, growth is definitely enhanced on serum or blood agar, or on boiled-blood agar.

The colonies on *ordinary agar* are small (1 mm. diameter) and very transparent, but under a low power of the microscope appear to have a compact finely granular centre and a pale transparent periphery. The appearances are similar to those of a culture of streptococcus, but the growth is slightly less vigorous; also the colonies are more transparent, watery, and flatter. '*Pneumococcus mucosus*' (*vide supra*) produces relatively large colonies of mucoid consistence.

Growing on *blood agar* the colonies are somewhat larger than on ordinary agar and the organism produces a greenish coloration of the medium, attributed to methæmoglobin formation. There is also partial hæmolysis. After about forty-eight hours the colonies often present a characteristic morphology, having a depressed centre and raised border, after further incubation their appearance is like that of a draughtsman with a central elevation and concentric raised ridges alternating with depressed zones from the centre to the periphery. Under anaerobic conditions complete hæmolysis may occur round the colonies without green coloration. The hæmolysin of the pneumococcus has been studied by Cowan who found that it appears in young cultures

coccus produces hydrogen peroxide, whilst there is only slight formation of catalase, the peroxide formed, not being destroyed by catalase, is a factor in inhibiting growth and in the self-killing of cultures (*vide infra*). The organism is active in producing either oxidation or reduction, according to

the tension of free oxygen present (Avery and Neill). The same processes occur in filtrates and lysates of cultures. Thus hæmoglobin is converted to methæmoglobin in the presence of oxygen, but at a low oxygen tension a reversal of this process takes place. In *broth*, growth forms at first a uniform turbidity but tends to settle at the bottom of the vessel as a granular deposit.

It is of interest that unheated tissues from certain plants when added to culture medium may accelerate growth, e.g. turnip, carrot, potato, etc. These may possibly act by neutralising the inhibitory effect of peroxide by virtue of their oxidising-reducing action (Avery and Morgan)

Colony variants. As in other bacterial species, variation associated with changes in colony characters, etc. is noted in cultures and especially when the organism is placed under unfavourable conditions. Thus a culture with the typical 'smooth' type of colony may undergo alteration to a 'rough' form, the colonies becoming thicker, heaped up, more opaque, more coherent, and with a granular surface. This change is associated with variation in other features which will be dealt with later.

BIOCHEMICAL CHARACTERS. The pneumococcus ferments glucose, lactose, saccharose, and usually raffinose, fermentative action on inulin is important, as failure of an organism to ferment this carbohydrate renders it most unlikely that it is a pneumococcus. Apparently some samples of inulin are less suitable for this reaction than others. Usually the test is carried out with Hiss's inulin serum water medium, in which coagulation of the serum results, but some investigators have found inulin broth more satisfactory, acid production being estimated by titration against sodium hydroxide with a phenolphthalein indicator. (*S. viridans* also frequently ferments inulin.)

Virulent pneumococci are soluble in bile. To demonstrate this, fresh ox bile autoclaved for twenty minutes at 120° C. and filtered, is added to a well-grown fluid culture (which must be one in simple broth) in the proportion of about a fifth of the culture. Two per cent. sodium taurocholate may be similarly used, but such solutions may be somewhat variable in their effect. It is supposed that bile acts by hastening the natural autolysis of the organisms. The reaction should be carried out at pH 7.6. It should be noted that 'rough' variants of the pneumococcus may sometimes be bile-insoluble.

Mair (1917) recommended as an improvement in the test the use of a 10 per cent. solution of sodium desoxycholate, alkaline to phenolphthalein. Of the solution 0.1 c.c. is added to 5 c.c. of a serum broth culture of the organism, clearing of the mixture occurs in ten to fifteen minutes. If the culture is acid, precipitation of the bile acids may occur, but this disappears on the addition of a drop or two of normal caustic soda.

VIABILITY Cultivation may be maintained for long periods if fresh subcultures are made every two or three days, but individual cultures die rapidly. They sometimes rapidly lose their virulence, so that four or five days after isolation from the body their pathogenic action disappears, but this is not always the case, especially if blood or serum media be used for maintaining subcultures, or if subcultures are made at very short intervals. The pneumococcus is comparatively sensitive to external agencies. The thermal death-point is about 52° C. In culture it soon dies when dried. When present in dry sputum or blood, however, it is more resistant and may retain its vitality for a considerable time. A convenient method to preserve the organism is to dry thoroughly the spleen of a mouse dead of pneumococcal septicæmia. In the dried tissue the organism not only remains alive, but may retain its virulence. The most satisfactory method of maintaining viable cultures is by rapid drying from the frozen state.

Autolysis. The pneumococcus readily undergoes spontaneous disintegration under certain conditions. Thus autolysis occurs in broth within the pH range 5.0 to 7.0, but is prevented when the acidity is greater than 5. The addition of fresh serum prevents lysis. When autolysates of cultures are added to suspensions of the pneumococcus in phosphate solutions of appropriate pH which have been heated at 60° C. for thirty minutes, lysis of the dead organisms is produced. The autolysis is associated with proteolytic and lipolytic action. Whether the self-killing is due to peroxide (*vide supra*) or to autolytic enzymes is open to question, but it is noteworthy that broth cultures of the pneumococcus die rapidly when in small bulk, *e.g.* 1 c.c.,



FIG. 19 Stroke culture of pneumococcus on blood agar. Twenty-four hours' growth at 37° C. Natural size



FIG. 20 Pneumococcus from a pure culture on blood agar of twenty-four hours' growth, some in pairs, some in short chains. Stained with dilute carbol-fuchsin $\times 1,000$

whereas they survive for several days in larger bulk, *e.g.* 5 c.c. in the same size of tube. Anaerobic conditions apparently favour survival, aerobic conditions promote autolysis.

RELATIONS TO STREPTOCOCCI. The facts that in cultures the pneumococcus often grows in chains, and that streptococci are potentially capsulate, have raised the question...

In identifying the

ters must be taken

tation of inulin, and behaviour with pneumococcus type sera are important

It may be stated, however, as bearing on the close relationships of the pneumococcus and streptococci, that Rosenow believes he has succeeded in transforming streptococci into capsulate organisms having all the biological features of the pneumococcus. Morgenroth and his co-workers, by treating pneumococci in various ways by optoquine, claim to have succeeded in effecting transformation into *S. viridans* and less frequently into a hemolytic streptococcus, a reversion was also met with, but only rarely. These results are of interest, but further investigation of the subject is required.

It is noteworthy that coccal types are met with which show characters intermediate between the true streptococci and the pneumococcus, *e.g.* Aronson's streptococcus (*vide p. 158*). Attention may also be drawn here to a group of cocci originally described by Schottmüller, isolated from various diseased conditions in man (pneumonia, otitis, meningitis, suppurations), these organisms possess voluminous capsules apparently consisting of a viscous material which gives a slimy consistence to cultures and also to pathological exudates. They are related to the pneumococcus on the one hand and to the streptococci on the other. The work of the Rockefeller Institute investigators (*vide infra*) suggests that these organisms ought to be classified into two groups: (1) The

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Pneumococcus mucosus (Fig 21): this organism tends to be less pointed than the pneumococcus, and its colonies are larger and more numerous than that of the typical pneumococcus (Fig 22). It is highly pathogenic to white mice (Fig 23), while the typical pneumococcus is not so pathogenic (Fig 24). (Fig 25) transverse section of the capsule in blood.



FIG. 21. *Pneumococcus mucosus* (Type III) from peritoneal fluid of mouse. Capsules stained $\times 1000$.



FIG. 22. *Streptococcus mucosus* in pus. Stained with Löffler's methylene blue. $\times 1000$.

the *Pneumococcus*

may be found

capsule

organism

Gulbransen)

under special conditions in the original host (Browning and

Toxic Products. Pneumococcal infections, and particularly acute pneumonia, show signs of pronounced toxæmia, but studies of the organism have not succeeded in defining fully its toxic products. Reference has been made to the hæmolysin, which may be of the nature of an exotoxin; it is oxygen-sensitive like one of the hæmolysins of *Strept. pyogenes*. A 'leucocidin' has been demonstrated (Oram), but its similarity to the leucocidins of staphylococci and streptococci is doubtful. The production of a necrotising filterable substance has also been reported by Parker; it is sensitive to oxidation. The capsular polysaccharide (p. 192), though it acts as an aggressin, is non-toxic *per se*. In culture and also in the tissues a product is liberated from the organism which produces a reaction with reduction of methylene blue, and which is probably a cleavage product of the capsular polysaccharide.

The oxygen-carrying capacity of the blood. Further work is still required to elucidate the complete toxic mechanism of the organism.

Experimental Inoculation. The pneumococcus is pathogenic to various animals, though the effects vary somewhat with the serological type and the virulence of the strain used. The susceptibility of different animal

species, as Gamaleia showed, varies to a considerable extent. The rabbit, and especially the mouse, are very susceptible; the guinea-pig, the rat, the dog, and the sheep occupy an intermediate position, the pigeon is immune. In the more susceptible animals the general type of the disease produced is not pneumonia, but a *septicæmia*. Thus, if a rabbit or a mouse be injected subcutaneously with pneumonic sputum, death occurs in from twenty-four to forty-eight hours. There is little inflammatory reaction at the point of inoculation, but the blood contains capsulate pneumococci in large numbers (Fig 23)

When relatively non-virulent cultures are used, local inflammatory changes are set up in susceptible animals instead of septicæmia, and corresponding results are said to follow when virulent cultures are injected into such animals partially immunised. So also in the more resistant animals, such as the sheep and the dog, the lesions produced are of inflammatory type, and when the injections are made into the lungs pneumonia may be set up. Lamar and Meltzer by intra-bronchial insufflation of cultures of pneumococci in the dog, succeeded in producing typical lobar pneumonia; although, in the cases where recovery followed, the general course of the infection did not fully correspond with that in the human subject. Comparatively large amounts of culture were used. More conclusive experiments on the subject, however, are those of Blake and Cecil who, by intratracheal injection in monkeys of virulent pneumococci in minute doses, were able regularly to set up a lobar pneumonia which ran a course like that of the human disease, recovery taking place by crisis. On the other hand, the introduction in larger amounts of similar pneumococci into the throat or nose of these animals was not followed by pneumonia, even though the organisms persisted for a considerable time. This is of importance in connection with the natural occurrence of pneumonia, as it points to some additional factor being necessary for its origin, especially in view of the fact that monkeys are apparently more susceptible than the human subject. Blake and Cecil met with spontaneous pneumonia in monkeys kept in confinement, and the disease, which had the same features as that experimentally produced, readily passed from animal to animal. They came to the conclusion that when virulent pneumococci are introduced into the trachea they probably penetrate the wall of a large bronchus near the root of the lung, and thence spread by the interstitial tissue outwards from the hilum, a general inflammation of the substance of the lung is thus produced. They were unable to set up lobar pneumonia in monkeys by subcutaneous or by intravenous injection, on the other hand, secondary invasion of the blood stream by the pneumococci occurred in the experimentally produced lobar pneumonia, the number of organisms increasing in cases which terminated fatally. McLeod and Gordon have failed to reproduce Blake and Cecil's results, although a fatal general infection occurred.

Various workers have pointed out that in lobar pneumonia spread of the infection in the lung takes place from one alveolus to another, the organisms

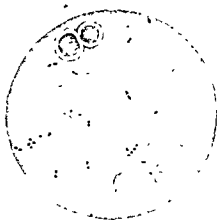


FIG. 23. Capsulate pneumococci in blood taken from the heart of a rabbit, dead after inoculation with pneumonic sputum. Dried film, fixed with corrosive sublimate. Stained with dilute carbol-fuchsin $\times 1,000$

being carried by the œdema fluid at the periphery of the advancing inflammatory lesion (Loeschcke; Gunn and Nungester; and others); and, from the results of experiments on monkeys, Loosli assumed that spread occurred *via* the bronchioles by aspiration in the act of breathing. Nungester and his associates have shown that in rats inoculated with pneumococci along with mucin by the nasal or tracheal route pneumonia only occurred in the presence of an additional factor, such as forced inspiration or alcoholic intoxication—the latter interfering with the physiological mechanism which prevents aspiration of fluid material into the lung. Such results throw further light on the etiology of the human disease.

Certain other experimental observations are noteworthy. Gaskell, administering adjusted doses by the intratracheal method, found that the forms of pneumonia thus produced in the rabbit varied according to the virulence. With a certain strain a lobular pneumonia might result, while with one of higher virulence there was lobar consolidation. Goodner has found in rabbits that after the dermal inoculation of virulent organisms a characteristic inflammatory skin lesion develops and this may resolve by 'crisis', though it may be succeeded by a fatal septicæmia.

The experiments of Webster and Clow are of special interest in relation to the pathogenicity of the pneumococcus and the epidemiology of infection by this organism. They found that the pneumococcus freshly isolated from the human subject and instilled in small doses into the nose of mice brought about a characteristic infection which spread to healthy contacts, producing in them either the carrier state or a fatal infection. The difference in pathogenic effects among individual animals ranged from the refractory state or a local cervical lymphadenitis to fatal lobular or lobar pneumonia with or without pleurisy, empyema, pericarditis, or septicæmia. Considerable differences were also noted among strains as regards their virulence on intranasal instillation.

The Occurrence of the Pneumococcus in Pneumonia and other Conditions.

The pneumococcus occurs in every variety of the disease—in acute lobar pneumonia, in broncho-pneumonia, and in septic pneumonia. In a case of lobar pneumonia the pneumococcus is found all through the affected area in the lung, especially in the exudate in the air cells. It also is present in the pleural exudate, and in the lymphatics of the lung. The greatest numbers are found in the parts where the inflammatory process is most recent. When the inflammation is resolving, some of the organisms often stain badly (e.g. tend to lose the Gram-positive reaction); such individuals are probably either dead or degenerate. Sometimes there occur in pneumonic consolidation areas of suppurative softening, which may spread diffusely. In such areas the pneumococcus occurs with or without ordinary pyogenic organisms, streptococci being the commonest concomitants. In other cases, especially when the condition is secondary to influenza, gangrene may supervene and lead to destruction of large portions of the lung. In these a great variety of bacteria, both aerobes and anaerobes, are to be found. By direct extension to neighbouring parts, empyema, pericarditis, and lymphatic enlargements in the mediastinum and neck may take place; the organism may occur either alone or with pyogenic cocci. In over three thousand cases of acute lobar pneumonia Heffron recorded the relative incidence of the pneumococcus and other organisms as follows. pneumococcus, 96·1 per cent.; streptococci, 2·8 per cent., pneumobacillus, 0·5 per cent., influenza bacillus, 0·2 per cent.; staphylococci,

pneumococcus
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this organism in the saliva of healthy persons. It can certainly be isolated by inoculation of susceptible animals, from the mouths of a considerable proportion of normal persons, from their nasal cavities, etc., being probably in any particular individual more numerous at some times than at others (especially, it is stated, during the winter months). It was reported, however, by Rockefeller Institute workers (*vide infra*) that the pneumococcus occurring in the healthy naso-pharynx is usually of Group IV, i.e. the least pathogenic to man. The more pathogenic types are found almost exclusively in the mouths of convalescents and of contacts and in rooms where pneumonia cases have been and certain persons

infection are still imperfectly understood, but the importance of predisposing causes must be recognised in the etiology of the disease. Such factors as cold, alcoholic excess, abnormal conditions of the respiratory tract—a slight bronchitis, etc.—may play an important part in the causation of pneumonia by lowering natural resistance to pneumococcal infection or by interfering with the normal physiological mechanisms which protect the pulmonary tissue from invasion by bacteria from the upper respiratory passages.

In a considerable proportion of cases of lobar pneumonia, the pneumococcus can be cultivated from the blood. This usually represents a bacteraemia rather than a true septicaemia and is due to a secondary extension of organisms from the lung, and varies in degree in different cases. Generally the tendency to pronounced bacteraemia is correlated with the age of the patient, being much greater over forty years of age than in younger persons. When bacteraemia is marked the disease is liable to have a severe course, thus the presence of over one hundred pneumococci per c.c. indicates a grave prognosis even in cases treated with chemotherapeutic drugs. It is interesting to note that in the lobar pneumonia experimentally produced by Blake and Cecil in monkeys, a progressive increase in number of pneumococci in the blood was accompanied by a progressive diminution in the number of leucocytes and led to a fatal result. The presence of the pneumococcus in the blood explains the occurrence of such inflammatory complications as meningitis, suppurations in connective tissue, joints, etc. However, a primary meningitis, apart from pneumonia, may be produced by the pneumococcus (*vide infra*). Ulcerative endocarditis may also develop, sometimes after the occurrence of a distinct crisis, and in this connection we may refer to the interesting observation made by Mair (1923), namely, that in rabbits partially immunised by dead cultures intravenous injection of virulent pneumococci is followed by endocarditis in a large proportion of cases. The organisms obtained from the vegetations were found to have lost their virulence.

Other forms of pneumonia and other primary pneumococcal infections
The so-called 'simple' or non-suppurative form of broncho-pneumonia common in children shows certain points of difference from the lobar form. It represents a pneumonia starting as multiple foci, usually in both lungs, and is the result of spread of inflammatory change from the terminal bronchioles along the air passages to the alveoli, although extension through the walls of the bronchioles also takes place. There is an absence of the rapid general involvement of the interstitial tissue of the lung seen in lobar pneumonia, and an important point is that it is characterised by the absence of crisis. In broncho-pneumonia in children the pneumococcus is again an important causal agent and can be found in many cases. Other organisms of the pyogenic group may occur along with it or sometimes alone. *B. influenza*

is also frequently found. In the adult the proportion of cases caused by organisms other than the pneumococcus is somewhat greater. In the broncho-pneumonia secondary to diphtheria the pneumococcus may be accompanied by the diphtheria bacillus and also by pyogenic cocci. In typhoid pneumonia the typhoid bacillus may be alone present or may be associated with the pneumococcus. In septic pneumonia the pyogenic cocci in many cases are the only organisms discoverable, but the pneumococcus also may be present. In influenza there is a lowering of the resistance of the bronchial mucosa and a great variety of organisms, including *B. influenzae*, pneumococci, streptococci, staphylococci, *Diplococcus catarrhalis*, etc., are able to extend to the lower bronchial passages. The bacterial flora present in the broncho-pneumonic lesions therefore varies considerably, but the pneumococcus and influenza bacillus are predominant and are often present together. In very severe influenza, such as occurred in the 1918 epidemic, in addition to broncho-pneumonia, lobar pneumonia due to the pneumococcus occurred, and sometimes even a true septicaemia due to this organism—an indication of remarkably lowered resistance. We may note that empyema due to the pneumococcus is more frequent in children than in adults, being a not uncommon sequel of broncho-pneumonia.

It is difficult to explain why sometimes the pneumococcus is associated with a spreading inflammation as in lobar pneumonia, while at other times it is chiefly localised in the patches of broncho-pneumonia. It is quite likely that in the former condition the organism is possessed of a different order of virulence, and probably the susceptibility of the lung tissue varies at different periods of life—the resulting lesion, of course, depends on both of these factors. There is, however, a closely analogous fact in the case of erysipelas, this being a spreading lesion produced by a streptococcus, which when less virulent causes only local inflammatory and suppurative conditions. As already mentioned, the occurrence of a crisis in lobar pneumonia, indicating a rapidly developed immunity, is a characteristic feature, and the association of this disease with a rapid growth of the organisms throughout the framework of the lung has undoubtedly an important significance. When the organisms are mainly present within the air vesicles and bronchioles, as in broncho-pneumonia, this rapid immunity reaction is not met with.

In children the pneumococcus may extend along the Eustachian tubes and give rise to otitis media, this being an important lesion; thence it may spread to the middle ear. It should be noted that next to the lobar pneumonia attack by the pneumococcus, usually secondary to a form of pneumonia or otitis media, but sometimes as a primary occurrence. Other primary pneumococcal lesions (apart from pneumonia), such as arthritis, peritonitis, etc., are also met with. Primary pneumococcal peritonitis, which frequently affects young female subjects, is often due in such cases to infection reaching the peritoneum from the vagina (McCartney). Not infrequently rhinitis is due to the pneumococcus, as are also inflammatory and suppurative lesions in the maxillary antrum and frontal sinus. Conjunctivitis is sometimes caused by the pneumococcus, as is also corneal ulcer.

Immunity to Pneumococcal Infection. Lobar pneumonia represents an inflammatory lesion accompanied by the symptoms of an acute poisoning. In very few cases does death take place from the functions of the lungs being interfered with to such an extent as to cause asphyxia. On the other hand, in fatal cases there may be profound toxic effects on the heart and nervous system. These considerations, along with the fact that the organisms are in greatest numbers in the lung, indicate the toxic mechanism which is operative

during the disease. But active immunity developed in the course of the infection or as a result of artificial immunisation is not a function of specific antitoxins; it is *antibacterial*, and is due to the development of powers to resist the invading organisms. Also, acquired immunity is type-specific, *i.e.* resistance is acquired only for the serological type (*vide infra*) which constitutes the immunising stimulus.

Animals can be immunised against the pneumococcus by inoculation with virulent cultures killed by heating at 55° C, with autolysed cultures or with cultures which have become attenuated in various ways. Sometimes one or two injections, at intervals of several days, are sufficient for immunisation, but the immunity has been observed to be usually of a fleeting character and may not last more than a few weeks. The serum of such immunised animals, when added to the organisms, neutralises the pathogenic action of the pneumococcus in susceptible animals; it protects also against subsequent inoculation with a certain dose of the pneumococcus, and if injected within twenty-four hours after inoculation, may even prevent death.

Pneumococcal infection is, however, one of the conditions to which a more effective immunity is developed by inoculating with living than with dead organisms. Neufeld and Rimpau insisted that the ultimate use of living virulent cultures was necessary for the attainment of a high degree of immunity. Blake and Cecil in their work on experimental pneumonia in monkeys likewise found that small doses of living virulent pneumococcus or larger doses of the same organism in an attenuated state protected against intratracheal injection of the virulent pneumococcus, while vaccines consisting of dead organisms failed to do so. The latter, however, modified the secondary invasion of the blood when pneumonia developed, and also afforded protection against experimental pneumococcal septicæmia. There was thus a humoral immunity without protection against intratracheal infection. They pointed out, however, that these results did not mean that dead vaccines might not protect the human subject against pneumonia under natural conditions, in view of the fact that the monkey is more susceptible than man. Moreover, we have evidence that dead culture vaccines do afford a degree of protection. The use of the living virulent organism is, of course, difficult to control, and is impracticable as a preventive measure against natural pneumonia in the human subject.

The protective potency of an anti-pneumococcal serum as measured by the number of fatal doses of virulent pneumococci against which a given amount of serum will protect an animal (*e.g.* a mouse), may reach a high

large number of organisms may occur. Armstrong has studied quantitatively the development of pneumococcal antibodies and has found, for

geometrical progression. The increase then becomes less rapid, the maximum concentration occurring about the eighth day. He finds further that in a case of lobar pneumonia running an ordinary favourable course, similar phenomena are met with. A rapid outpouring of antibodies occurs about the fifth day, and the content in the serum increases till about the time of the crisis, after which it remains high for some time. We thus have the picture of a rapid development of active immunity, which on reaching a certain point results in a rapid destruction of the organisms.

An anti-pneumococcal serum exhibits various antibody reactions, including agglutination and precipitation. There is no evidence, however, that an antiserum, in virtue of its antibodies, possesses bactericidal action. The organism is sensitive, however, to opsonic action, and thus an antiserum may exert its beneficial action by means of its opsonins. Observations have been carried out on the opsonic properties of the serum in cases of pneumonia, and it has been found that towards the time of crisis in favourable cases the opsonic index rises distinctly, and after deservescence gradually returns to normal. Blake and Cecil in their work on experimental pneumonia in monkeys did not consider it possible to explain the immunity by the antibodies demonstrable in the serum, and considered that some other factor such as changes locally in the tissues, might be concerned in the immunity reaction and the destruction of the organisms. Sia, Robertson and Woo have shown that the immunity acquired during the illness is associated with an increased power of the serum to promote the killing of the pneumococcus by a rabbit serum-leucocyte mixture. At the crisis they found this property markedly developed, and absent in fatal cases. These results tend to emphasise the importance of opsonic antibodies in immunity to the pneumococcus. According to H. D. Wright's observations (1927) on immunised rabbits, the property possessed by the whole blood of delaying growth of virulent pneumococci *in vitro*, is the manifestation of antibody activity which appears earliest and lasts longest.

The leucocytosis in pneumonia has long been recognised, and when present in distinct degree indicates that a satisfactory cellular response is taking place. It, however, scarcely indicates more than this, and certainly does not promise recovery, as death may occur in a variety of ways. On the other hand, the absence of leucocytosis, or its disappearance after being present, is undoubtedly of unfavourable omen.

It may be noted here in conclusion, that in man immunity against pneumoma may be short-lived, as in many cases of pneumonia a history of a previous attack is elicited. It is, of course, possible that a second attack may be due to a type of pneumococcus differing immunologically from that of the first infection (*vide infra*). If this were so, any immunity persisting from the earlier disease would not be protective, as the immunity mechanism is specific for each type of the organism.

Differentiation of Types of the Pneumococcus by Antisera. This has been one of the important consequences of the study of immunity against the pneumococcus. It had been long recognised that strains of the pneumococcus derived from different sources present individual peculiarities, but it was not till the exhaustive investigation of the subject in the Rockefeller Institute, New York, that definite results were obtained. In the study of the agglutinating and protective properties of antisera prepared by inoculating animals against a long series of cultures isolated from cases of acute lobar pneumonia, it was proved that sera derived from certain strains, on the one hand, would almost indiscriminately agglutinate some of these strains, and, on the other, had little or no effect on other strains. It was further found that the agglutinating and protective qualities of these sera were parallel. In this way it was possible to subdivide the strains into four types. Three of them (I, II, III) were serologically well defined, and a fourth (IV) was formed of strains in which an antiserum usually agglutinated only the strain which originated it, and had little or no capacity of agglutinating the strains of Types I, II, or III. The members of Type III were recognised not only by developing agglutinating sera specific for the type, but since they presented cultural features which characterised them as the *Pneumococcus*

mucosus Types I and II together accounted for 60 per cent. of the cases of pneumonia studied and were of relatively high virulence for man, this being specially the case with Type II. Type III, while accounting for only 12 per cent. of cases, was of highest virulence, the mortality with it being 45 per cent. Type IV (more appropriately designated Group IV) was found in 24 per cent. of cases and caused the lowest mortality (16 per cent.), the strains occurring in the mouths of healthy individuals were found to belong mainly to this group. However, in different parts of the world different types prevail. Thus, in South Africa, Lister found that, while the New York Types I and II were common, nearly a third of all cases of pneumonia were associated with another type which apparently did not occur to any extent in New York. Among a variety of pneumococcal infections (studied in Edinburgh in 1924-25) Type I predominated and was present in 64 per cent. of all cases, while Type II was relatively infrequent (7 per cent.). Type III and Group IV represented respectively 15 per cent. and 14 per cent. of the infections. It is interesting to note that the type-incidence may vary at different times in the same area: thus in Edinburgh during 1929-30 Type II was the most prevalent variety, causing 40 per cent. of cases of lobar pneumonia, while Type I occurred in 29 per cent. The death-rate in Types I and II cases respectively was 23 and 31 per cent. (Alston and Stewart). Types I and II are apparently more invasive than Type III or Group IV (R. Cruickshank), as illustrated by the prevalence of I and II in primary meningitis, peritonitis, and empyema, and the frequency of the others in otitis media, secondary meningitis, and conjunctivitis. As a result of an extensive inquiry into the relative prevalence in lobar pneumonia of Types I, II, III, and Group IV in various parts of the world, Heffron reported the following percentages: Type I, 32.8, Type II, 20.6, Type III, 10.8, Group IV, 35.8. Little significant difference was noted between relative prevalence in North America and Europe, but as mentioned above, the distribution of types is somewhat different in Africa, Types I and II being less frequent and Group IV more common. Group IV is largely responsible for primary broncho-pneumonia, the infection being an endogenous one (R. Cruickshank). Blacklock and Guthrie in a study of lobar and broncho-pneumonia among infants and children found that 90.7 per cent. of pneumococcus strains from these conditions belonged to Group IV.

The constituent serological types of Group IV were later classified by Cooper and her co-workers in America. Twenty-nine types were identified (designated IV to XXXII), and only a small proportion of strains remained unclassified. Certain types were found to be more prevalent than others in lobar pneumonia of adults. The majority of the types were also recognised in normal persons and in other infections of the respiratory tract.

or even III, the majority individually showed a very low prevalence (under 2 per cent.). The following summary (quoted from White) states the general position, the types being given in order of frequency:

Lobar pneumonia

Infants and children	XIV, I, VI, V, VII
Adults	I, II, III, VIII, VII, V.

Bronchio-pneumonia

Infants and Children	VI, XIX, XVIII
Adults	III, VIII, XVIII, X, V, VII

In Edinburgh (1938-44) among the types of Group IV none has been found to approach in prevalence Types I, II, or even III, and it could not be said that particular types of Group IV showed special prevalence though Type VIII was the most frequent, with Type VI second in order. Among a small group of cases of broncho-pneumonia in young subjects Types XIX and VI were the commonest; this corresponds with American findings. As regards Types III and VIII, Anderson has found that in the West of Scotland these were present in the sputum of over 40 per cent. of persons in whom the only respiratory abnormality was a slight bronchitis. In this area the invasive types, in addition to I and II, were V and VII.

Recently our knowledge of the serological classification of pneumococcus strains has been considerably extended. As pointed out by Kauffmann and others cross-reactions may be observed in serological typing and exact determination of a type may only be possible by means of sera absorbed with cross-reacting strains. On this basis new types have been identified additional to those designated by Cooper and her associates. Walter and others described seventeen new types by means of reciprocal antibody-absorption tests, these were first numbered in sequence with Cooper's types but later were correlated with new types reported by Kauffmann. Still later Eddy proposed that all new types should be designated separately in numerical sequence, irrespective of their relationships, and described in all seventy-five types under Arabic figures in place of the original Roman numerals. She also showed that most pneumococcus types give some reaction with one or more heterologous sera and that individual types behave so specifically in these cross-reactions that two types which may appear to be identical when tested with one serum can be separately identified by their heterologous reactions. Eddy has suggested a system of grouping of pneumococcus types for the preparation of polyvalent therapeutic and diagnostic sera. Fourteen groups are recommended on the basis of the antigenic relationships of the constituent types. Mørch at Copenhagen has also made valuable contributions to the serological classification of the pneumococci.

THE SPECIFIC SUBSTANCES OF THE DIFFERENT TYPES OF PNEUMOCOCCUS. In 1917 Dochez and Avery noted in the blood and urine of cases of lobar pneumonia a substance which gave a precipitation reaction with an antiserum for the type of pneumococcus causing the infection. It could be detected by mixing equal quantities of clear centrifuged urine and an appropriate antiserum. The appearance of this substance in the urine depends on the severity of the case, and a progressive increase in amount is significant from a prognostic standpoint. It was observed also in young cultures, and apparently diffuses readily from the organisms into the surrounding medium. Heidelberger and Avery isolated this specific substance from Type II pneumococcus. They used a method of fractional precipitation by alcohol, acetone and ammonium sulphate, and dialysis. It appeared to be a polysaccharide yielding 79 per cent of reducing sugars on hydrolysis, and gave no protein reactions. Though it did not act as an antigen *in vivo*, it was able to react in a specific manner *in vitro* with a homologous anti-pneumococcal serum. In its immunological relations it belongs to the group of 'haptens' (Landsteiner). An immunologically and chemically identical polysaccharide has been demonstrated in yeast and in one serological type of the pneumobacillus. Similar polysaccharides were later obtained from Type III and Type I pneumococci, but all proved to be chemically different substances, each reacting specifically with the corresponding antiserum. The precipitin reactions elicited with these bodies occurred sometimes in very high dilutions. The Type I substance was found to contain nitrogen as part of its molecule.

These specific substances have been extensively studied by various workers from the chemical and immunological standpoints, and the facts show that their formation is intimately related to the capsulation and the virulence of the organisms. Strains which have undergone 'rough' variation and lost virulence, also show a loss of their capacity to form their specific carbohydrate. Associated with these changes there is an absence of type-specific characters and capsule formation. These carbohydrate substances also produce cutaneous reactions in specifically immunised animals or persons (Francis). In addition to this type-specific polysaccharide 'haptén', the pneumococcus also contains a protein antigen which is not type-specific but common to all types; thus, avirulent 'rough' variants lacking the type-specific 'haptén' contain this protein antigen. A species- or group-specific polysaccharide has also been isolated.

It has been shown by Sia that the specific substance added to a serum-leucocyte mixture annuls the bactericidal effect of the latter. This effect has also been found to be specific, thus, the soluble carbohydrate of Type I pneumococcus interferes specifically with the bactericidal action of whole blood towards this type. This property may be an important factor in pneumococcal infection, and the specific polysaccharide of the organism possesses 'aggrassin' functions. It is not toxic *per se*. The specific effect on the bactericidal power of whole blood may be due to its union with and fixation of opsonic substances. The transformation of one type of pneumococcus to another type by inoculating a 'rough' derivative of the former along with a dead culture of the latter type has been recorded by Griffith and others. Thus an 'R' variant may acquire the power to form a polysaccharide specifically different from that possessed by the original strain. According to observations of Alloway, an R variant derived from a type-specific S form may be changed *in vitro* by growth in broth containing anti-R serum along with a heated filtered extract of an S-form pneumococcus of different type, and thus becomes a 'rough' variant which the e

of type apr
It is chemically quite distinct from the capsular carbohydrate which is developed under its influence (Avery, MacLeod and McCarty). These results are of great biological interest.

In addition to the type-specific polysaccharides referred to above, various other non-protein substances have been separated which may enter into the antigenic composition of the pneumococcus.

Avery and Dubos have discovered an enzyme extracted from a saprophytic bacterium which is capable of hydrolysing the capsular carbohydrate of the pneumococcus. Type III pneumococcus acted on by this enzyme is rendered avirulent and the enzyme has been found to exert a protective action *in vivo* in mice against infection by this organism.

It is of interest that Type XIV pneumococcus and the erythrocytes of the human blood group A show an antigenic similarity dependent apparently on similar carbohydrate haptens, thus human erythrocytes may be agglutinated by a Type XIV antiserum (Weil and Sherman, Deeson and Goebel).

METHODS OF CLASSIFYING PNEUMOCOCCI BY SEROLOGICAL REACTIONS. The original method for identifying Types I, II, and III, and Group IV was briefly as follows. Specific agglutinating antisera for the three types respectively (I, II, III) were used. A white mouse was inoculated intraperitoneally with 0.5 to 1 c.c. of a saline emulsion of a bean-sized piece of sputum, preferably quite fresh, freed from surface contamination by washing in sterile saline. When the mouse died in from five to twenty-four hours, if the peritoneal exudate contained a strong and fairly pure growth of the pneumococcus the abdominal cavity was washed out with 5 c.c. saline, cultures being at the same time made in broth and on blood agar plates. The peritoneal washings were first centrifuged

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SPECIFIC SUBSTANCES OF THE DIFFERENT TYPES OF PNEUMOCOCCUS.

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In the therapeutic application of the serum in a case of Type I pneumonia large and repeated doses had to be used, and it was therefore a necessary preliminary to determine whether the patient exhibited supersensitiveness to horse serum, and to desensitise him if this existed (*vide p 136*). Very soon after commencement of treatment the temperature often rose, but in many cases this was quickly succeeded by a fall, with improvement in the patient's general condition, stoppage of extension of the lung lesion, and prevention of invasion of the blood by the pneumococci. The effects of the treatment proved satisfactory—of 107 cases treated in the Rockefeller Institute Hospital up to October 1917 only 7.5 per cent. died, as compared with a mortality of 25 to 30 per cent. in cases

water or weakly acid buffer solutions. This enabled an effective dose to be given intravenously in relatively small bulk and with less frequent and marked reactions due to the protein injected (Park, Bullowa and Rosenbluth). The concentrated serum had five to ten times the potency of the unrefined serum. Subsequently Felton (1931) introduced an improved method of concentrating the serum, alcohol being used in this case as the precipitant, and this method was generally adopted. Repeated doses of 10,000 to 40,000 Felton units (*vide infra*) were administered. The results indicated a pronounced efficacy of the serum in Type I infections, and successful results were also obtained with mono-specific sera in pneumonia due to Types II, V, VII, VIII, and XIV. It must be emphasised that an important factor in determining the value of serum treatment is the stage of the infection at which it is commenced, early administration is essential.

It has also been reported that unconcentrated antisera produced by immunisation of rabbits may be superior to sera obtained from the horse. Such rabbit sera seem to be quantitatively as active as a concentrated horse antiserum. It has further been pointed out that the antibody molecule from the rabbit is smaller than that from the horse and thus penetrates more readily into infected tissue. Clinical evidence apparently supported these claims regarding the therapeutic value of rabbit antisera (see Horsfall *et al*, Goodner *et al*, 1936).

Since the introduction of chemotherapy of pneumococcal disease by sulphonamides or penicillin, serum therapy has been little used, though it has been suggested that a combination of chemotherapy and serum therapy may in some cases give better results than one of these forms of treatment by itself.

Standardisation of Anti-pneumococcal Serum. In general the basis of standardisation has been the protective value of the serum against virulent pneumococci injected into mice, the quantitative tests being made by mixing varying dilutions of a pneumococcus culture with a given quantity of serum, varying quantities of serum with a given number of pneumococci. The latter is the system adopted by Felton whose unit of potency is the amount of serum which protects a mouse against a million fatal doses of pneumococcus culture. The titration of a new serum is carried out by comparison with a standard serum.

PROPHYLACTIC VACCINATION. In the South African mines a special situation existed in consequence of the great susceptibility to pneumonia arising in the native labourers, who were chiefly recruited from sub-tropical regions. As the case incidence was from 30 to 150 per thousand per annum, and the mortality from 10 to 30 per thousand, the disease became a serious problem. Almoth Wright introduced prophylactic vaccination, after, founding on his investigations (*vide supra*), prepared a vaccine containing the three prevalent types of the pneumococcus. Three injections at ten-day intervals of, in all, 7,000 million cocci, killed by an antiseptic, were administered. A very marked diminution in the incidence of the disease followed. Cecil and Austin in the United States recorded the results of vaccinating about 10 per cent. of 32,000 men in a camp with a mixture of

Types I, II, and III, two to four doses being given. In the subsequent period of ten weeks no cases of pneumonia due to these types developed among those vaccinated, while among the unvaccinated there were twenty-six cases.

It has been shown recently that immunity in man can be produced by subcutaneous injection of a single dose of the capsular polysaccharide: thus MacLeod and his co-workers have found that immunisation with 0.03 to 0.06 mgm. of each of the polysaccharides of types I, II, V, and VII is effective in preventing pneumonia caused by these types, the immunity being, of course, type-specific. Protection develops within two weeks and lasts for at least six months.

Bacteriological Diagnosis. In stained films of sputum, pus, or other exudate containing pneumococci, the outstanding feature is the predominance of diplococcal forms, the elements of which may have a lanceolate shape and which are Gram-positive. The capsule may often be observed as an unstained zone round the diplococci; and this is confirmed by relief staining. A special capsule stain may of course be used, but is not generally required for routine purposes. Cultures on blood agar (preferably heated blood agar) should be made and after twenty-four hours at 37° C. will, if the pneumococcus be present, show characteristic colonies. Bile-solubility and the reaction with inulin should be tested with pure growths obtained from single colonies. A mouse may be inoculated intraperitoneally to test the pathogenicity and to afford in blood films corroborative evidence of capsulation. The serological type of the pneumococcus can also be determined by the methods described above. After treatment with penicillin or sulphonamide drugs it may not be possible to demonstrate the pneumococcus, e.g. in the cerebro-spinal fluid in cases of meningitis, either by microscopic examination or culture.

Blood culture should be carried out in cases of pneumonia to ascertain whether a general infection is present, and the result, as has been explained, may be of prognostic value. Further information is obtained by repeated blood culture: for this purpose a fixed quantity, e.g. 1 c.c., or preferably varying quantities are added to melted agar, and the number of colonies is counted after incubation. In this way a progressively increasing blood infection can be recognised. When a case has been under sulphonamide therapy 1 per cent. *p*-aminobenzoic acid should be incorporated in the medium (*vide* Appendix).

Particularly in early cases of pneumonia sputum may not be obtainable for bacteriological examination and for the typing of the organism. By puncture of the affected lobe in a majority of such cases material is obtained for examination of the organism present (Stewart).

length attached to a 20 c.c. Record syringe.

few drops of blood-stained fluid are aspirated. The fluid is mixed with part of which is incubated, while part can be utilised for inoculation of a mouse

CHAPTER VI

THE MENINGOCOCCUS AND GONOCOCCUS AND ALLIED ORGANISMS (NEISSERIEÆ)

MENINGOCOCCUS (*NEISSERIA INTRACELLULARIS*)

SINCE the beginning of the nineteenth century the occurrence of epidemics of cerebro-spinal meningitis has been recognised. As the result of observations on this disease in different parts of the world, it has been established that the causal agent is the organism first described by Weichselbaum and named *Diplococcus intracellularis meningitidis* but now usually spoken of as the meningococcus. Cases occur chiefly from infancy to young adult age, while the sporadic form of posterior basal meningitis among young children is also due to this organism. Epidemics are met with in military barracks and camps among young soldiers. There may be an associated septicæmia. A septicæmia without meningeal infection may also be due to the meningococcus and this condition may pursue a subacute or chronic course as well as occurring in a fulminating form. In the last condition, bilateral adrenal hæmorrhage is often a feature.

MICROSCOPIC CHARACTERS. The meningococcus is an oval coccus measuring about 0.8 to 1 μ in diameter, it usually occurs in pairs, the adjacent surfaces being somewhat flattened, or even slightly concave. Sometimes tetrads are observed and the cocci may occur also in larger aggregates. In many cases the organisms are chiefly contained within polymorphonuclear leucocytes in the exudate, e.g. in the cerebro-spinal fluid (Fig. 21), some-



FIG. 21. Film preparation of exudate from a case of meningitis, showing the meningococci within leucocytes. Stained by Gram's method and dilute carbol-fuchsin $\times 1,000$.

times, however, the majority may be lying free, and this is especially the case in virulent infections. The meningococcus stains readily with basic aniline dyes, but is Gram-negative. It may be noted that the cocci frequently show metachromatic granules demonstrable by staining with Löffler's methylene blue and also by Neisser's method as applied to the diphtheria bacillus. Capsules have been noted in some strains. Both in appearance and in its staining reactions it is closely similar to the gonococcus. In artificial cultures the typical shape and arrangement may be absent, and enlarged involution forms are seen, especially in older growths, which may stain deeply or faintly.

CULTURAL CHARACTERS. The organism can readily be cultivated aerobically outside the body, anaerobic conditions are unfavourable, additional carbon dioxide promotes growth. But in primary cultures and when recently isolated it usually fails to grow on ordinary meat infusion peptone agar unless there is an admixture of serum, ascitic fluid or blood (fresh or heated). However, agar medium prepared with meat digest (provided it has not been deprived of growth factors by filtration) supports growth, boiled blood

digest agar is recommended for obtaining profuse cultures. The optimum reaction is pH 7.0 to 7.4. Growth takes place best at the temperature of the body, and practically ceases at 25° C. The colonies are circular disks with a slightly opaque centre fading into a delicate transparent margin (Fig. 25), and exhibit a smooth, shining surface; they have a slightly mucoid consistence and readily emulsify in water or normal saline. When examined under a low magnification the centre appears somewhat yellowish, and the margins usually are smooth and quite regular; at a later period of growth slight crenation may appear, especially when the medium is somewhat dry. The colonies may be of considerable size, attaining a diameter of 2 to 3 mm. on the second day. After

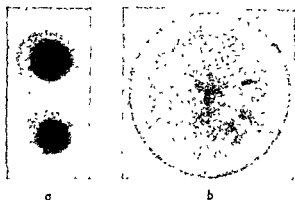


FIG. 25. *a* Two-days' colonies of the meningococcus, $\times 0$. *b* the same, in which illumination has been arranged to show finely granular centre and transparent margin $\times 12$. Compare with Fig. 30 (From photographs by the late Prof W. B. M. Martin)

adaptation to artificial cultivation, growth may be obtained without serum in the medium. On horse blood agar hæmolytic is observed. Variations in appearance are met with, *e.g.* the development of R (rough) and other forms, a minute rough form and a large mucoid variant have been described. In serum broth the organism produces a general turbidity with formation of some deposit after a day or two; but growth in fluid media is not abundant. In primary cultivations, especially when the organisms are scanty, growths may be best obtained by incubating several c.c. of the cerebro-spinal fluid with an equal volume of glucose broth.

In the preparation of a medium suitable for culture purposes most attention has been directed to the readiness with which growth takes place and to the abundance of the growth, but, as pointed out by Murray and Ayrton, retention of the virulence of the organism should also be a criterion of suitability. They have found that retention of virulence depends upon substances contributed by the digest used as a basis, and they have also made the interesting observation that the addition of an extract of polymorphonuclear leucocytes *in vitro* raises the virulence (For the formulae of their media their original papers must be consulted). It has been shown also that, according to the medium used, the virulence may be lowered and raised several times in succession.

BIOCHEMICAL REACTIONS. The meningococcus ferments glucose and maltose with acid production, properties which distinguish it from *Diplococcus catarrhalis* (*vide infra*); and it has no action on lactose, saccharose, or inulin. Strains have been observed which do not ferment maltose. Fermentation tests can be carried out by means of either fluid or solid media containing serum and 1 per cent. of the sugar to be tested, along with neutral-red or litmus as an indicator.



FIG. 26. Pure culture of meningococcus, showing coccoid forms. From a 48 hours' culture. Stained by Gram's method and dilute carbol-fuchsin. $\times 1,000$

VIABILITY The life of the organism in cultures is a comparatively short one, after a few days cultures will often be found to be dead, but by making subcultures every three or four days strains can be maintained alive for considerable periods. Cultures survive longer at 37° C than at room temperature. On egg medium, however, the organism survives for a considerable time. It is readily killed by heat at 55° C., and is also very sensitive to weak antiseptics, drying for a few hours has been found to be lethal to it. Cultures can be preserved in the viable state for long periods when rapidly dried *in vacuo*. The facts established accordingly show the meningococcus to be a somewhat delicate parasite.

Reference has been made to the enlarged involution forms observed in cultures after a few days' incubation, and these are also seen to undergo subsequent disintegration. This is due to an autolytic enzyme as originally shown by Flexner, who found that it also has the property of lysing other bacteria.

Distribution in the Disease and Pathogenic Effects. The organism occurs in the exudate in the meninges and in the cerebro-spinal fluid, and it can usually be demonstrated in the fluid obtained by lumbar puncture. In acute cases, especially in the earlier stages, it is usually abundant, but in the later stages of cases of more subacute character, its detection may be a matter of difficulty, and only a few cocci may be found after a prolonged microscopic search. But it should be recognised that *at any stage* microscopic examination and even cultivation may sometimes give a negative result. In most cases the lesions are practically restricted to the nervous system, but occasionally complications occur, and in these the organism may be present. It has been found, for example, in arthritis, pericarditis, pneumonic patches in the lung, and in other inflammatory conditions associated with the disease, and also in purpuric patches in the skin and stomach. It may be obtained from the blood during life. A blood infection may also occur without obvious meningeal lesions and such cases may run a chronic course.

Experimental inoculation shows that the ordinary laboratory animals are relatively insusceptible to this organism. An inflammatory condition may be produced in mice and guinea-pigs by intraperitoneal injection, and a fatal result may follow. The results vary according to the virulence, but often the organism does not undergo very active multiplication, though it may sometimes be cultivated from the blood, none of the lesions in the nervous system is reproduced. Similar results are produced by dead cultures, and occasionally the lethal dose of the dead organisms may equal that of the living (Gordon). There is thus evidence that an active toxin plays an important part in the pathology of the disease. Flexner and also Stuart McDonald have shown that cerebro-spinal meningitis may be produced in monkeys by injections of the organism into the spinal canal, the latter observer finding that exudate containing meningococci was more effective than cultures. In such experiments the organisms extend upwards to the brain, and produce meningitis within a very short time. The resulting lesions, both as regards their distribution and general characters, and also as regards the histological changes, resemble the disease in the human subject. Even these animals, however, are manifestly less susceptible than the human subject. Meningitis has been produced in rabbits by intracisternal injection of virulent meningococci, a similar effect resulted from the introduction of filtered suspensions, and even boiled culture produced histological changes in the meninges similar to those in an infective meningitis (Branham and Lillie). Such findings may be regarded as manifestations of the toxic action of the organisms. It is noteworthy that when chicken embryos are

inoculated with a suitable strain of meningococcus the organism localises specifically in the meninges and causes an acute meningitis similar to that found in man (Buddingh and Polk).

The interesting observation has been made by Miller that even a minute dose of meningococcus culture if suspended in a solution of gastric mucin can produce a rapidly fatal infection on intraperitoneal injection in mice. Moreover, the animals show a pronounced general infection, the organisms being demonstrable in the circulating blood. An egg-yolk emulsion has apparently a similar effect to that of mucin (Sacharow).

Toxic Products. The claim has been made that the meningococcus produces an exotoxin (Ferry, Norton and Steele). It has been stated that when grown in flasks in a suitable broth (pH 6.6) a pellicle forms and within a few days a soluble toxin can be demonstrated, which in high dilutions produces skin reactions in animals and which incites antitoxin formation. Ferry has used this antitoxin in therapeutic tests in infected monkeys and states that it is effective in preventing a fatal result. According to Malcolm and White it is possible to extract a highly toxic substance from cultures but they regard it as an endotoxin. Other workers have also found that an antiserum may neutralise certain toxic preparations obtained from the meningococcus. The high toxicity of the organism is undoubted and there is some evidence that the toxin is antitoxinogenic but only weakly so, as compared with the antitoxinogenic power of the true exotoxins. It must be concluded that the toxic products of the organism are of the nature of endotoxin, though these may be rapidly liberated from the bacterial cell when death with autolysis takes place.

Mode of Infection. The meningococcus can usually be found in the naso-pharynx of patients suffering from the disease, and there is no doubt that this is the usual site of invasion, infection being conveyed by secretion droplets from the mouth, throat, and nose. In cases where recovery occurs, the organism may persist there for a varying period of time—usually only for a week or two, but sometimes for months. There is difference of opinion as to the route by which the organism passes from the naso-pharynx to the meninges. One view is that it passes directly by the lymphatics to the base of the brain, but satisfactory evidence of this is wanting. The other view is that it reaches the meninges by the blood stream; this is in accordance with what occurs in other infections, and is supported by the fact that in some very acute cases with purpuric eruption, it has been found in the blood before meningeal symptoms have appeared. It also occurs occasionally in septicæmic cases without meningitis. For a considerable time it has been known that contacts of cases of cerebro-spinal fever often harbour the meningococcus in the naso-pharynx, that is, are 'carriers', and during the war of 1914-18 this subject was extensively investigated. The proportion of carriers among contacts varies, 20 per cent or higher. Non-contacts have been examined during epidemics, and among them also a considerable proportion, though not so great as among contacts, have been found to be carriers. Among troops during the war of 1914-18 the normal carrier rate was about 2 to 4 per cent. In a report by the Ministry of Health (1930) it was clearly shown that a certain proportion of meningococcal carriers may be found in the normal population in the series of observations dealt with, 2.44 per cent. of the persons examined were carrying the organism. In troops it was noted that just prior to an epidemic of cerebro-spinal meningitis the carrier rate increased and after it reached 20 per cent., or over, cases of the active disease appeared. It was also observed that in overcrowded quarters the carrier rate was high, whereas the spacing out of the beds effected a marked decrease

in the percentage of carriers. Thus overcrowding is one of the most active factors in promoting the spread of the disease and it has been generally accepted that the occurrence of an epidemic is conditioned by the prevalence of carriers. Dudley and Brennan have shown, however, that a high carrier rate is not necessarily accompanied by cases of meningitis, they have recorded in one instance a carrier rate of 50 per cent extending over a period of a year without associated meningitis. In some carriers the meningococcus occurs sparsely among other organisms, but in others in fairly large proportion, and occasionally in almost pure culture. In the great majority of carriers the organism can be found for only a comparatively short time—a few days even, or a week or two—but in a small proportion it persists for months, these being 'chronic' carriers. Unfortunately, we have at present no ready means of estimating the relative virulence of meningococcus strains obtained from the naso-pharynx and from elsewhere. With regard to the epidemiology certain facts are outstanding. Direct infection (with resulting meningitis) of a healthy individual from a patient suffering from the disease is comparatively uncommon, though it sometimes occurs, and it is rare for a known carrier to develop the disease. On the other hand, there is substantial evidence of persons being infected from carriers. The facts mentioned would indicate that the organism is spread widely from individual to individual, in most cases without obvious effects, but that when the organism reaches a susceptible individual the disease may rapidly develop, in this connection the high susceptibility of young children must be remembered. No doubt the number of the organisms in the naso-pharynx of the carrier is a factor of importance. It has been stated by some observers that the presence of the meningococcus leads to, or is associated with, naso-pharyngeal catarrh, and that this often precedes meningeal infection. In fact, the primary lesion may be a naso-pharyngitis. On the other hand, the organism may abound in the naso-pharynx without the presence of catarrh or any abnormality. Manifestly the act of coughing will aid in its diffusion when it is present.

Apart from the epidemic form of the disease, cases of a sporadic nature also occur, in which the lesions are of the same nature, and in which the meningococcus is present. The facts stated would indicate that the origin and spread of the disease in the epidemic form depend on certain unknown conditions which produce an increased virulence of the organism.

Identification of the Meningococcus and Serological Types. In cases of meningitis this usually presents no difficulty, as the finding of a Gram-negative diplococcus in the cerebro-spinal fluid is practically conclusive. When the organism occurs in the naso-pharynx, however, the matter is quite different. Means must be taken to distinguish it from other Gram-negative diplococci which occur in this situation. Originally, the following points taken together were usually accepted as justifying a diagnosis: *conformity in the microscopic characters and in the appearance of the colonies with those of the meningococcus, ready emulsification in saline, absence of growth at room temperature, fermentation of glucose and maltose, and non-fermentation of saccharose*. Attempts have been made to identify the meningococcus by means of agglutination reactions. Dopter was the first to show that strains obtained from the naso-pharynx, in other respects conforming to the meningococcus, might not be agglutinated by the serum of an animal immunised against a known strain of this organism, he applied the term '*parameningococcus*' to such strains. Gordon found that a serum prepared by injecting any one strain did not agglutinate all the strains isolated from the cerebro-spinal

fluid and naso-pharynx Proceeding further, he arrived finally at the recognition of four 'types' (I-IV), according to agglutinating tests, cross-agglutination between them being little marked (see Gordon and Murray). Types I and II were the commonest, the latter being rather the more frequent. All the strains separated from cases of meningitis were found to be agglutinated by one of the four sera. Gordon's results were criticised by Griffith and by Fildes, by one coccus

Fildes also stated that while the rabbit tends to produce a monospecific serum on being inoculated with a strain of meningococcus, the horse when similarly inoculated may produce a serum which is less specific. It appears hardly justifiable, therefore, to conclude that an organism may not be the meningococcus merely because it is not agglutinated by certain sera, provided that it conforms in other respects.

Griffith classified meningococci serologically on a different basis from that of Gordon. He divided them into two groups (I and II) by direct agglutination tests. Group I embraced Gordon's Types I and III, and Group II included Types II and IV. According to Griffith, Group I is more complex in antigenic structure than Group II. He found also that strains isolated from the pharynx could be similarly classified, but only a minority belonged to Group I, and suggested a relationship between virulence and antigenic complexity. Other workers have concluded that increase of virulence is associated with greater complexity in antigenic constitution. Single colonies from a particular strain may vary in antigenic structure as shown by Maegraith who points out that Types I and III are not fixed and that there is an overlap between them. Branham in the United States, who also found that Types I and III are difficult to separate, originally noted overlapping between II and IV, though in later years these types became more distinct. Segal has reported the occurrence of a 'Type V' meningococcus. Rough variants are non-specific in their antigenic characters. Antisera protect mice against a fatal infection with meningococci of homologous types.

In summary it may be said that the meningococcus exhibits considerable antigenic variation. For practical purposes of diagnosis it may be divided into two groups (comprising four types), of which Group I is more commonly pathogenic, but meningococci of other antigenic constitution may be capable of pathogenic action in man.

Chemical studies of the antigenic constituents of these organisms have indicated the presence of three components, one a carbohydrate which is common to the group, another a protein which is highly toxic, and a third, a type-specific substance, which in Types I and III is of carbohydrate nature, but in Type II is associated with protein (see Rake and Scherp, Menzel and Rake). Strains which have been repeatedly subcultured on artificial media tend to lose their type-specific characters.

An interesting method of eliciting precipitation reactions with the meningococcus has been devised by Petrie, who grows the organism on solid medium in which an antiserum is incorporated. The occurrence of a halo round the colony denotes the production of precipitate, constituents of the organism having diffused from the growth into the surrounding medium and reacted with the homologous antibody.

Preparation of agglutinating sera The following method has been used successfully. A rabbit receives on one day three intravenous injections of 500 millions of organisms killed at 55° C., with an interval of an hour between the injections, six days afterwards it receives a single dose of 2,000 millions. Eight days later the serum has usually a titre

of over 1:800. Young rabbits of about a kilogram in weight give the best results. For testing, four dilutions are used, namely 1:50, 1:100, 1:200, 1:400. Emulsions of known type organisms are tested as controls at the same time. After the mixtures are made they are placed at 55° C for twenty-four hours, and the results are then read.

Precipitin reaction with the cerebro-spinal fluid in cases of meningitis. Rake has shown that the reaction of meninges is very sensitive for infection. A ring of opacity develops in the tube containing the corresponding antiserum.

about the same time, and though they are not always proportional in amount to the agglutinins, the two classes of substances have more or less the same significance, and may occasionally be of use in diagnosis when lumbar puncture fails to give positive results. Although their presence in large amount may be said to indicate a marked reaction, they do not supply information of much value in relation to prognosis. Immune bodies, as shown by bactericidal and complement fixation tests may also be developed in considerable amount in the course of the disease. On the other hand, carriers appear not to develop antibodies in their serum.

Therapeutic Antisera. Before the introduction of the sulphonamides which have proved highly successful in the treatment of meningococcal meningitis, serum therapy was mainly relied on as a standard method of treatment. Early results offered great promise of the efficacy of this form of therapy, but later findings have not confirmed the original successes. The discrepancy between earlier and later results has not been explained. The type of serum which was most extensively used was that originally prepared by Flexner and Jobling. The serum was obtained from the horse by repeated injections of dead cultures in increasing doses, followed by culture autolysate and living cultures, these two latter being best administered by the subcutaneous route. Several strains of the meningococcus were mixed together for purposes of injection, and the immunisation was continued over a period of several months. For treatment, anti-meningococcal serum was injected under the spinal dura, 30 c.c. being generally used as the dose for an adult, this being repeated on subsequent days, or even twice daily. Some of the spinal fluid was removed, and then the serum was injected, undue pressure being avoided. Intravenous injections were combined with the intrathecal administration. This serum was used on a large scale in various parts of the world, and there was originally general agreement as to its favourable effects—thus it was claimed that the mortality of the disease, generally 70 to 80 per cent, was reduced to about 30 per cent or even less, and the tendency to the occurrence of chronic lesions was also markedly diminished. Later results, however, especially in the treatment of sporadic cases of meningococcal meningitis in children proved unsatisfactory. The mode of action of such antisera was not fully explained. They contained opsonins, agglutinins, and antibodies which fixed complement. After the injection the numbers of cocci were apparently reduced, probably as a result of increased phagocytosis, there could scarcely be any direct bactericidal action owing to the absence of complement. During the war of 1914-18 monovalent sera against each of the four types of meningococcus and also a polyvalent serum were prepared for military cases by Gordon and his co-workers. The standardisation of such antisera proved a matter of some difficulty, at first the fixation of complement method was used, but later the opsonic index was regarded with more

favour as a criterion of the potency of the serum. Gordon stated that the serum also possessed antitoxic properties towards the endotoxin of the organism, and he pointed out the importance of estimating its antitoxic action. The agglutination reaction and a protection test in animals were also advocated for assaying the serum. It is doubtful if any of these methods provided a reliable means of estimating the therapeutic potency of anti-meningococcal sera and in all probability different antisera and different batches of serum prepared by the same method varied considerably in this respect.

Allied Commensal Diplococci. In the naso-pharynx there occur commensal Gram-negative diplococci which morphologically have a close resemblance to the meningococcus and show other characteristics. Such commensal diplococci are classified into two main subgroups: those which completely lack saccharolytic or fermentative properties, exemplified by the *Diplococcus*

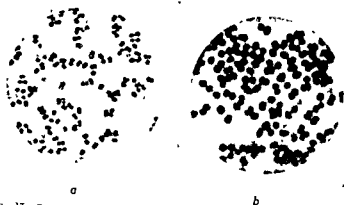


FIG. 27. *D. catarrhalis* -
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catarrhalis, and those possessing fermentative properties exemplified by the type known as *Diplococcus pharyngis flavus*. The latter subgroup collectively might be named *D. pharyngis*. Certain specific or type designations have been given to these organisms in the literature but it is questionable whether such separate names are all justifiable and whether the reputed species or types have been clearly differentiated. Many of the characters on which specific or type differentiation have been based are probably dependent on fluctuating variation.

Diplococcus (or *Micrococcus*) *catarrhalis* (*Neisseria catarrhalis*). In addition to occurring in health this organism has been found in large numbers in catarrhal conditions of the pharynx and respiratory passages. Its microscopic appearances are practically similar to those of the meningococcus, and it may also be found within leucocytes. Some strains are characterised by the large size of the cocci (Fig. 27). The colonies on serum agar, though on the whole tending to be rather larger and more opaque, closely resemble those of the meningococcus, but are somewhat tough in consistence and not so readily emulsifiable. The features of colonies may show considerable variation and S and R variants have been described. The organism usually grows aerobically on ordinary media without serum, and at 20° C., and it has none of the fermentative properties described above as belonging to the meningococcus.

Diplococcus pharyngis flavus (*Neisseria flava*). Certain varieties under this designation have been distinguished by various writers but it cannot be said that a clear subdivision has been established. The microscopic characters are the same as those of *D. catarrhalis*. Young colonies may resemble those of the meningococcus but later they tend to assume a greenish-yellow colour. Growths may be obtained aerobically on ordinary media without serum and usually also at room temperature though the optimum is 37° C. A type has been described, however, which does not flourish at the lower temperature, but it can be distinguished readily from the meningococcus by the fermentation of saccharose. Strains are met with which simulate the meningococcus in fermentative reactions but these can be distinguished by their ability to grow at room temperature on ordinary media. The criteria detailed on p. 201 serve to differentiate all these organisms from the meningococcus.

Diplococcus pharyngis siccus (*Neisseria sicca*) grows at room temperature, and its colonies are very tough and adhere to the surface of the medium, it can thus be distinguished readily from the meningococcus. It has marked fermentative properties, acting on glucose, maltose, and saccharose. This organism is probably a 'rough' variant of another type of commensal diplococcus. The *Diplococcus mucosus* has colonies of slimy consistence, it grows at room temperature, and forms capsules (*vide infra*) which can be demonstrated by the method of Hiss or by relief staining. This designated type probably represents a variant of one of the other members of the group. An organism called the *Diplococcus crassus* is also met with, it is rather larger than the meningococcus, and, especially in subcultures, may tend to assume staphylococcal forms. Its Gram staining is variable, both positive and negative individuals being often seen in the same culture. It grows on ordinary media and at room temperature. The colonies are very small and not unlike those of a streptococcus. Glucose, maltose, lactose, and saccharose are fermented.

Occasionally organisms referable to the group described above and clearly distinguishable in biological characters from the meningococcus, have been found in cases of meningitis. Branham (1930) in a small outbreak of meningitis in America, isolated from the cerebro-spinal fluid in a proportion of cases an undescribed meningococcus-like organism (*Neisseria flavescens*) which, growir
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agglutinated by antisera to any of the four types of meningococci. An organism resembling *Diplococcus mucosus* has also been described in meningitis (see Edwards). It ferments glucose but not maltose or saccharose.

Meningitis due to other organisms. Meningitis may be produced by almost any of the pyogenic organisms. A considerable number of cases, especially in children, are due to the pneumococcus. In many instances

infrequently in lobar pneumonia, and in some cases with cerebral symptoms we have found it present where there was merely a condition of congestion. Occasionally epidemics of meningitis have been due to the pneumococcus. Sporadic cases are also met with, and this organism comes next in order of frequency to the meningococcus as the cause of primary meningitis. The pneumobacillus also has been found in a few cases. Meningitis is not infrequently produced by streptococci, especially when middle-ear disease is

present, and less frequently by staphylococci; occasionally more than one organism may be concerned. In meningitis following influenza the influenza bacillus has been found in a few instances, but sometimes the pneumococcus is the causal agent. Sporadic cases of meningitis occur associated with organisms which resemble the influenza bacillus morphologically and also in presenting hæmophilic culture reactions, but which possess marked pathogenic properties for rabbits and guinea-pigs. Both in the cerebro-spinal fluid and in cultures, these bacilli frequently show a tendency to produce long filamentous forms and also may show a beading of the protoplasm. The cases from which such bacilli have been isolated have chiefly occurred in children, are extremely fatal, and may follow on an otitis media, from which condition similar organisms have been isolated. Sometimes the meningitis is part of a septicæmia or pyæmic process—in the latter the joints are often affected. The biological relationships of the organisms associated with such conditions are dealt with in a later chapter. They certainly tend to be more widely distributed in the body of the infected individual than is the case in influenza. An invasion of the meninges by the anthrax bacillus occurs, but is a rare condition; it is attended by diffuse hæmorrhage in the sub-arachnoid space. In tuberculous meningitis the tubercle bacillus, of course, is present, especially in the nodules along the sheaths of the vessels.

Various other pathogenic organisms have on occasions been reported as producing meningitis, e.g. gonococcus, *B. typhosus* and *Salmonella* organisms, *B. abortus*, *Actinomyces*, meningitis may be a feature of infection by the poliomyelitis virus and cases of meningitis have been described which are due to another filterable virus (*vide* lymphocytic choriomeningitis).

In conclusion, it may be stated that mixed infections may occur in meningitis. Thus the pneumococcus has been found associated with the tubercle bacillus and also with the meningococcus, sometimes appearing as an additional infection to the latter.

Bacteriological Diagnosis of Meningococcus Infection. During life the methods involve the microscopic examination of the centrifuged cerebro-spinal fluid and the making of cultures therefrom. For the former, films stained by methylene blue and by Gram's method make the recognition of the meningococcus relatively easy in many cases, and the presence of Gram-negative diplococci, especially within cells, is practically diagnostic. In addition, plates of boiled blood digest agar are inoculated; this should be done without delay as the organisms tend to die in the course of a few hours when the fluid has been kept at room temperature. The meningococcus when cultivated can be identified by its characteristic cultural and biochemical features and by agglutination tests with group or type antisera. Cultures of the organism may be obtained even when microscopic examination has given negative results, and it is always advisable to mix several c.c. of the fluid with an equal volume of 1 per cent glucose-broth and incubate at 37° C., since in this way cultures can be obtained when no growth results from direct plating.

Sometimes difficulty arises in diagnosis when no organisms are detectable microscopically or by the cultivation test. Here the character of the exudate may afford some indication of the nature of the infection. A predominance of polymorphonuclear cells is usually manifest in meningococcal, pneumococcal, and *B. influenzae* meningitis, whereas in tuberculous meningitis the exudate is as a rule chiefly lymphocytic. In pneumococcal and *B. influenzae* infections the organisms are usually detectable by culture on blood agar, if not observed microscopically. To speak generally, if with a polymorph

exudate no growth is obtained on blood agar, the condition is most likely to be due to the meningococcus. Two other considerations must be borne in mind, if the meningitis is high up in the cerebro-spinal system, leucocytes may be present in the spinal fluid before organisms themselves are seen; and if a case has been treated with a sulphonamide compound or penicillin before bacteriological examination is carried out, it may be difficult, if not impossible, to demonstrate the meningococcus either by microscopic examination or cultivation. It should also be noted that a polymorph exudate may be observed in the spinal fluid in cases of poliomyelitis.

Meningococcal septicæmia may be demonstrated by blood culture, if the case has been treated with a sulphonamide compound 1 per cent of *p*-aminobenzoic acid should be incorporated in the medium.

Reference has been made earlier to the agglutination reaction with patients' serum which may be resorted to in cases where the organism cannot be demonstrated.

For the examination of the naso-pharynx a post-nasal swab is used, this is obtained by means of a West's tube to avoid contamination with organisms in the saliva. The necessary precautions must be taken to ensure immediate inoculation and incubation of plates, boiled blood digest agar being used as the culture medium. The resulting growths will, of course, be mixed, and meningococcus colonies may not be numerous among the colonies of other organisms. The various colonies are carefully scrutinised and Gram-stained films made from those which correspond to the meningococcus, and if they consist of Gram-negative cocci subcultures are made. The pure growths thus obtained are examined further as regards their cultural, biochemical and serological characters, commensal Gram-negative cocci being carefully excluded.

The detection of meningococcus carriers was at one time extensively relied on with a view to controlling the disease, but owing to the very high carrier rates among contacts, the segregation of proved carriers has often been impracticable, and the labour involved in identifying them has not been justified by the practical results.

The meningococcus can sometimes be demonstrated in the larger petechial lesions of the skin when a purpuric eruption is present. For this purpose material from the lesion is 'scooped out' with a Hagedorn or hypodermic needle and films made on several slides. These are stained by Gram's method and the characteristic Gram-negative cocci may be observed inside cells. This is recommended by some workers as a rapid means of diagnosis when there is a purpuric eruption (see Muir, and Tompkins).

GONOCOCCUS (*NEISSERIA GONORRHOÆE*)

The organism known to be the cause of gonorrhœa, and called the gonococcus, was first described by Neisser, who in 1879 gave an account of its microscopic characters as seen in the pus of gonorrhœal infections, both of the urethra and of the conjunctiva. He considered that this organism was peculiar to the disease, and that its characters were distinctive. Later it was successfully isolated and cultivated on coagulated human serum by Bumm and others. By inoculation of cultures in the human subject its causal relationship to the disease has been conclusively established.

MICROSCOPIC CHARACTERS. The organism of gonorrhœa is an oval coccus (about 0.8 to 1 μ in diameter) which usually is seen in diplococcal form, the adjacent margins of the two cocci being flattened, or even slightly concave, so that between them there is a small oval unstained interval.

An appearance is thus presented which has been compared to that of two beans placed side by side (Fig. 29). When division takes place in the two

commoner than as tetrads. Cocci in process of degeneration are seen as spherical elements of varying size, some being considerably swollen and staining faintly.

These organisms are found in large numbers in the pus of acute gonorrhœa, both in the male and female, and for the most part are contained within the leucocytes. In the earliest stage, when the secretion is glairy, a considerable number are lying free, or are adhering to the surface of desquamated epithelial cells, but when it becomes purulent the large proportion within leucocytes is a very striking feature. In the leucocytes they lie within the



FIG 28. Gonococci, from a pure culture on blood agar of twenty-four hours' growth. Some cocci already are beginning to show the swollen appearance common in older cultures. Stained with carbol-thionin $\times 1,000$.



FIG 29. Portion of film of gonorrhœal pus, showing the characteristic arrangement of the gonococci within leucocytes. Stained by Gram's method and dilute carbol-fuchsin $\times 1,000$.

protoplasm, especially superficially, and are often so numerous that the leucocytes appear to be filled with them, and their nuclei are obscured. It has been observed that the marked and characteristic phagocytosis of the organism occurs more in the purulent exudate than in the inflamed tissue. As the disease becomes more chronic the cocci gradually become fewer, though even in long-standing cases they may still be found in considerable numbers.

In artificial culture, the organisms have similar microscopic characters to those observed in the disease, but show a great tendency to undergo degeneration, varying in size—some being enlarged—and staining faintly and irregularly. This is specially so in cultures after incubation for four or five days. The less suitable the medium the more rapidly does involution take place. The gonococcus stains readily and deeply with a watery solution of any of the basic aniline dyes—methylene blue, fuchsin, etc. It is, however, easily decolorised, and is Gram-negative—an important point in microscopic diagnosis.

CULTURAL CHARACTERS. Cultivation is attended with some difficulty, as the conditions of growth are somewhat restricted. Blood or serum incorporated in the culture medium is necessary, and a variety of media have been advocated by different workers for the cultivation of the organism. One of the most suitable for routine culture is 10 per cent. heated blood agar, the meat infusion incorporated in it being prepared by the method recommended by Wright (1933) (p. 181). Nutrient agar with 10 per cent. ascitic

or hydrocele fluid may also be used. The optimum pH is from 7.3 to 7.6. It is advisable to inoculate the medium within half an hour after obtaining the material from the body, and to place the tubes or plates at once in the incubator. Stuart has shown recently, however, that if material containing gonococci is kept under conditions preventing oxidation the survival of the organisms at room temperature is greatly prolonged, so that cultures may be obtained after upwards of twenty-four hours (p. 215). Growth takes place best at 35° to 36° C (Jenkins), and ceases altogether at 25° C. The organism requires abundant moisture for its growth. Torrey and Buckell have advocated the use of a semi-solid agar in place of the ordinary solid medium. If tubed medium is employed, there should be sufficient condensation fluid present. A suitably moist atmosphere for the growth of the gonococcus can be obtained by incubating tubes or plates in an airtight jar containing a pad of wet glass-wool (Tulloch). Most satisfactory results are obtained by incubating the culture in an atmosphere with 8 per cent carbon dioxide. Liver extract and egg media have also been successfully used in the cultivation of the gonococcus. Cultures are obtained by taking some pus on the loop of the platinum wire and inoculating plates of medium by successive



FIG. 30. Colonies of gonococcus on serum agar, (a) three days' growth (b) and (c) five days' growth $\times 9$
(From photographs by the late Prof. W. B. M. Martin.)

strokes. The colonies are usually visible within forty-eight hours, and often within twenty-four hours; it is important, however, to note that sometimes growth may not appear till the fourth day. Different strains seem to vary in their ability to grow on artificial media in primary culture. The colonies are small, semi-transparent, rounded disks, and vary somewhat in size. They tend to remain more or less separate. Later, the margin may become undulate and the centre more opaque; radial and concentric markings may be present (Fig. 30). The first cultures die somewhat quickly, but in subcultures, kept at 37° C, the organism remains alive for a considerable time, sometimes three weeks. After about a week, more active foci of growth may appear in some of the colonies in the form of papillae. Atkin has drawn attention to two types of colonies which apparently represent different biological types of the organism: (I) large, irregular, thin colonies with papillae; (II) smaller, rounder, thicker, and denser colonies without papillae. He claims that the papillae of Type I are more viable than their substrate, and that Type II colonies are derived from these papillae of Type I.

McLeod and his co-workers have found that the recognition of gonococcus colonies is aided by the use of the direct oxidase reaction. Cultures are made on heated blood agar plates and after two days' incubation are covered with a 1 per cent solution of tetramethyl-*p*-phenylenediamine hydrochloride which is immediately run off. Medium-sized convex and translucent colonies which rapidly turn purple, may be accepted for diagnostic purposes as the

gonococcus if they consist of Gram-negative diplococci which show the characteristic fermentation reactions and do not grow on ordinary agar medium

The special requirements for the growth of the gonococcus have been studied by various workers. Certain amino-acids are essential, but McLeod *et al.* have shown that others may exert an inhibitory action, depending on their concentration and the presence of blood. Thus *D*-alanine and taurine, while inhibitory in certain concentrations, favour growth if 0.5 per cent. of blood is added to the medium. Whole blood acts better than serum in this respect, and blood heated at 55° C. better than fresh blood. An important function, therefore, of blood added to culture medium is the neutralisation of any growth-inhibitory properties of particular amino-acids present in the commercial peptone used. Starch added to the medium may also act in the same way by neutralising inhibitory factors. It has been shown that glutathione as derived from meat infusion, yeast, etc., may be an important growth factor and that strains which require this factor are inhibited by cystine and cysteine (Gould). Other amino-acids which have been found to be inhibitory are glycine, tryptophane, and tyrosine. Some workers have stressed the value of adding 1 per cent. disodium hydrogen phosphate to the medium, this probably acts in virtue of its buffering property. Thomson has recommended the addition of glucose.

BIOCHEMICAL REACTIONS. In fermentative reactions glucose is the only carbohydrate, among those generally used, which is acted on by the gonococcus, lactose, saccharose, and maltose are not affected. For fermentation tests solid media in the form of slopes should be used; a digest basis is incorporated in place of meat infusion, 1 per cent. of the particular sugar, an indicator of acidity, and 5 per cent. sterile unheated serum from the guinea-pig or rabbit (horse serum is unsuitable owing to its glucose content)

Comparison with Meningococcus The morphological and cultural characters of the gonococcus and meningococcus are in many respects closely similar; the following points are of importance in distinguishing them biologically. The conditions of growth of the gonococcus are more restricted than those of the meningococcus. The gonococcus does not grow on the ordinary agar media, whereas the meningococcus may grow slightly after repeated subculture. The colonies of the latter are generally more opaque and have more regular margins than those of the gonococcus. The gonococcus ferments only glucose whereas the meningococcus usually ferments maltose also. It has been pointed out that the meningococcus differs from the gonococcus in its power to produce hæmolytic when growing on 5 per cent. horse blood agar (Joachimovits)

SEROLOGICAL TYPES A study of the immunity reactions of the gonococcus with antisera has elicited the fact that this species of organism is serologically homogeneous. While certain strains possess antigenic characters common to the species as a whole, it is not possible to subdivide gonococcus strains into sharply defined serological types. Also observed variations in individual antigenic properties. According to subacute gonorrhœa in the male are caused by one fairly well defined serological type. Atkin has subdivided strains into two serological types, one comprising most strains from the acute disease, the other mainly from chronic infections. He suggests the possibility of transformation of the first to the second type. It seems likely that two main types exist, with intermediate forms.

The antigenic constituents of the gonococcus have been studied recently by chemical methods and precipitin reactions as in similar studies of the pneumococcus (*q v.*) Nucleo-protein and polysaccharide factors have been separated and it would appear that certain constituents are common to this organism and to the meningococcus and the pneumococcus (Miller and Boor).

Relations to the Disease and Experimental Inoculation. The gonococcus is invariably present in the urethral discharge in gonorrhœa, and also in other parts of the genital tract when these are the seat of gonorrhœal infection. It is likewise present in gonorrhœal conjunctivitis, and has been found in some cases in the secondary infections of joints. Its presence in these different positions has been demonstrated not only by microscopic examination, but also by culture. From the description of the conditions of growth in culture it will be seen that life outside the body in natural conditions is impossible—a statement which corresponds with the clinical fact that the disease is always transmitted directly by contagion. Inoculation of pure cultures in the urethra of lower animals, and even of apes, is followed by no effect, and a similar statement can be made with regard to inoculation of gonorrhœal pus itself. In fact, hitherto it has been found impossible to reproduce the disease by any means in the lower animals. On a considerable number of occasions inoculations of pure cultures have been made in the human urethra, both in the case of the male and female, and the disease, with all its characteristic symptoms, has resulted. (Such experiments have been performed independently by Bumm, Wertheim, and others.) The causal relationship of the organism to the disease has therefore been completely established. The infection tends to persist, *e g.* the gonococcus has been isolated seven years after the original attack (Carpenter *et al*), thus the carrier state is set up.

Intraperitoneal injections of pure cultures of the gonococcus in white mice

connective tissue, but they appear to have little power of proliferation, they soon disappear, and the inflammatory condition does not spread. Injection of pure cultures into the joints of rabbits, dogs, and guinea-pigs causes an acute inflammation, which, however, soon subsides, whilst the gonococcus rapidly dies; a practically similar result is obtained when dead cultures are used. These experiments show that though the organism, when present in large numbers, can produce a certain amount of inflammatory change in these animals, it has little or no power of multiplying and spreading in their tissues.

Toxin of the Gonococcus. De Christmas cultivated the gonococcus in a mixture of 1 part of ascitic fluid and 3 parts of broth, and found that the fluid after twelve days' growth had toxic properties. At this period all the organisms were dead, and the fluid constituted the 'toxin'. The toxic substances were precipitated along with the proteins by alcohol, and the

case of animals it produced no effect, its introduction into the human urethra caused acute catarrh, attended with purulent discharge. He found that no tolerance to the toxin resulted after five successive injections at intervals. In a later publication he pointed out that the toxin on intracerebral injection

had marked effects; he also claimed to have produced an antitoxin. The production of a soluble toxin as described by De Christmas has not been confirmed by subsequent workers, though recently Clark, Ferry and Steele have stated that a soluble toxin can be demonstrated in gonococcal cultures. Such toxic products as have been described are probably of the nature of endotoxins.

Distribution in the Tissues. The gonococcus having been thus shown to be the direct cause of the disease, some additional facts may be given regarding its presence both in the primary and secondary lesions. In the human urethra the cocci penetrate the mucous membrane, passing chiefly between the epithelial cells, causing a loosening and desquamation of many of the latter and inflammatory reaction in the tissues below, attended with great increase of secretion. There occurs also a gradually increasing emigration of leucocytes, which take up a large number of the organisms. The gonococcus penetrates the subjacent connective tissue and is especially found, associated with extensive leucocytic emigration, around the lacunæ. Even when the cocci have disappeared from the urethral discharge, they may still be present in the deeper part of the mucous membrane of the urethra, and also in the prostate. The prostatic secretion may be examined by making pressure on the prostate from the rectum ('prostatic massage') when the patient has almost emptied his bladder, the secretion being afterwards discharged along with the remaining urine. In acute gonorrhœa there is often a considerable degree of inflammatory affection of the prostate and vesiculæ seminales, but it is difficult to say whether these conditions are always due to the presence of the gonococcus in the affected parts. A similar statement applies to the occurrence of orchitis and also of cystitis in the early stage of gonorrhœa. The organism has, however, been obtained in pure culture from peri-urethral abscesses and from epididymitis; it is likely that the latter condition, when occurring in gonorrhœa, is usually due to the actual presence of the gonococcus. During the more chronic stages other organisms tend to appear in the urethra, aid in maintaining the inflammatory condition, and may produce some of the secondary results. The pyogenic cocci, coliform bacilli, diphtheroid bacilli, etc., are often present, and may extend along the urethra to the bladder and set up cystitis, though this may be aided by the passage of a catheter. It may be mentioned here that Wertheim cultivated the gonococcus from a case of chronic gonorrhœa of two years' standing, and by inoculation in the human subject proved it to be still virulent.

In the disease in the female, the gonococcus is almost invariably present in the urethra, the situation affected next in frequency being the cervix uteri. It does not appear to infect the mucous membrane of the vagina of the adult unless some other abnormal condition be present, but it does so in the gonorrhœal vulvo-vaginitis of young subjects. It has also been found in suppuration in connection with Bartholin's glands, and sometimes produces an inflammatory condition of the mucous membrane of the body of the uterus. The gonococcus may also pass along the Fallopian tubes and produce inflam-

this condition, usually unassociated with other organisms. Further, in a small proportion of the cases in which the gonococcus has not been found, no organisms of any kind have been obtained from the pus, and in these cases it may have been originally present and has subsequently died. Lastly, it may pass to the peritoneum and produce peritonitis, which is usually of a local character.

In *gonorrhæal conjunctivitis* the mode in which the gonococcus spreads through the epithelium to the subjacent connective tissue is similar to what obtains in the case of the urethra. The relation to the leucocytes in the purulent secretion is also the same. Microscopic examination of the discharge in acute cases often gives positive evidence, and pure cultures may be readily obtained. As the condition becomes more chronic, the cocci are less numerous and a greater proportion of other organisms may be present. An infection of the conjunctiva in new-born children, *ophthalmia neonatorum*, which is often of very severe nature, may also be due to the gonococcus.

Joint affections, etc. The relations of the gonococcus to the sequelæ of gonorrhœa form a subject of great interest and importance. The following statements may be made with regard to them. First, in a certain number of cases of arthritis following gonorrhœa pure cultures of the gonococcus may be obtained. A similar statement applies to inflammation of the sheaths of tendons. Secondly, in a considerable proportion of cases no organisms have been found. It is possible, however, that in many of these the gonococcus may have been present in the synovial membrane, as it has been observed to be much more numerous in that situation than in the fluid. Thirdly, in some cases, especially in those associated with extensive suppuration, found to be the joints, owish tint.

subject on inoculation with the cultures obtained. In another case, in which pleurisy was present along with arthritis, the gonococcus was cultivated from the fluid in the pleural cavity. It seems possible that arthritis may also occur as a manifestation of allergy to the products of the organism, as in streptococcal infection (*vide* p 171). The existence of a *gonorrhæal endocarditis* has been established. Cases apparently of this nature, occurring in the course of gonorrhœa were at one time described without direct bacteriological evidence, but the complete bacteriological test has been satisfied in several instances. In one case Lenhartz produced gonorrhœa in the human subject by inoculation with the organisms obtained from the vegetations. A true *gonorrhæal septicæmia* has also been demonstrated, cultures of the gonococcus having been obtained from the blood during life on more than one occasion (Thayer and Lazear). Such occurrences, however, are rare. Gonococcal meningitis has been recorded but is a very rare complication.

Immunity and Serum Diagnosis. Immunity following recovery from the infection is usually transient. Specific antibodies have been demonstrated in the serum of persons infected with the gonococcus, *e.g.* agglutinin, and complement-fixing antibody, and the complement-fixation test has now

valent antigen must be used, consisting of a number of strains sufficient to represent the different serological races of the gonococcus. The complement-fixation reaction is of limited value, either for the diagnosis or exclusion of gonococcal infection in general; but positive results are significant in cases of considerable duration where there is evidence of dissemination of the organism.

Price recommends an antigen prepared as follows: the organism is grown on hydrocele agar in a Roux flask and washed off with 100 c.c. saline solution. 1 c.c. of normal

sodium hydroxide is added and the suspension is placed at 37° C. for two hours; this

centrifuging and suspended in 4 c.c. saline solution; the suspension is then adjusted to pH 7.5 by the addition of decinormal sodium hydroxide when, after shaking, the precipitate undergoes solution, 1 c.c. of 1 per cent. formal-saline is added and the fluid after filtration is used as the antigen, diluted 1 in 30 for the actual test. According to Price, in the first week of a gonorrhœal infection 27 per cent. of cases yield a positive reaction with this antigen and the percentage of reactors increases with the duration of the illness, 100 per cent. giving the reaction after five weeks. Repeated negative reactions after treatment have been regarded as indicative of cure.

Allergy. A cutaneous allergic reaction can be elicited in certain cases by the intradermal injection of killed organisms, e.g. gonococcus vaccine. It is negative in the early stage of the infection, and though frequently positive in the later stages, is not uniformly so. The specificity of the reaction is not complete.

Vaccines. Both gonorrhœa itself and the secondary infections have been treated by means of vaccines, but the results reported vary greatly. On the whole, most success has been obtained in the case of joint infections and allied conditions, though even here reports are contradictory. The initial dose employed has been usually about five million cocci, but care is necessary in starting the treatment, especially in the case of acute gonorrhœa.

Serum therapy. Specific antisera have been applied in the treatment of gonorrhœa, but the results have not been such as to encourage their general use.

Bacteriological Diagnosis. For microscopic examination films of discharge, etc., are stained by Gram's method, and it is advisable to put alongside the film a small quantity of culture of a staphylococcus as a control: this should stain deeply violet while the gonococcus in the film is stained only with the counterstain. The presence in the urethral discharge in the male subject of large numbers of diplococci having the characters, position, and staining reactions already described, is generally regarded as conclusive evidence that the case is one of gonorrhœa. For all practical purposes there is no other condition in which this sum-total of microscopic appearances is present in a case of urethritis of the adult male. It is of value, however, that confirmation of the microscopic findings should be obtained by cultivation and examination of the biochemical characters. Even in chronic cases the typical microscopic picture is sometimes well maintained and a presumptive diagnosis can be made in this way, but when the specific organism is in small number and other bacteria are numerous, microscopic diagnosis may be difficult, if not impossible. It must also be remembered that Gram-negative diplococci other than the gonococcus but with similar morphology, may occur on the genital mucous membranes and these may become more numerous as a result of various pathological conditions. Further, coccal organisms which are normally Gram-positive may undergo degenerative changes in discharges and lose their Gram reaction and these may also simulate the gonococcus. The difficulty is noticeable in gonorrhœal infections of the conjunctiva and vagina (see de Bord), and particularly in chronic gonorrhœa in the adult female subject. Gram-negative diplococci other than the gonococcus may also be found in inflammatory conditions in these situations apart altogether from gonorrhœa. In such cases the cultivation test is essential to establish conclusive proof of gonorrhœal infection. Moreover, cultivation may reveal the presence of the gonococcus when microscopic examination fails. In a case in the adult female, discharge should be taken with a bacteriological

wire loop from the urethra, or with the aid of a speculum from the cervix uteri, and both microscopic examination and cultivation carried out.

If the specific organisms are scanty cultivation may also present great difficulty as the growth will be a mixed one with gonococcus colonies in a small minority. The direct oxidase reaction may facilitate recognition of colonies, but other organisms cultivated from these cases may give the reaction. It should be noted that the gonococcus can be cultivated in some cases from the centrifuged urine, and it has been claimed that this method in males is more satisfactory than cultivation from discharge, since it reveals the presence of the organism if it is located in the posterior urethra (see Allison *et al.*). 'Prostatic massage' may also be applied as a preliminary to bacterio-

It is of interest in regard to the diagnosis of gonorrhœa that cases of meningococcal conjunctivitis and vulvo-vaginitis have been reported, and that the gonococcus has been found, though very rarely, in meningitis (see Armytage).

Method of preserving Gonococci in material for delayed cultivation. Stuart recommends the following: to 100 c.c. of a hot previously autoclaved, solution of 0.3 per cent. fibre agar in water 0.2 c.c. thioglycolic acid is added, and the mixture is approximately neutralised with N/1 NaOH, then 10 c.c. of 20 per cent. sodium glycerophosphate solution in distilled water and 2 c.c. of 1 per cent. CaCl₂ are added. After thorough mixing, the medium while hot is adjusted to pH 7.4 with N/1 NaOH and 0.4 c.c. of 0.1 per cent. aqueous methylene blue added. About 7 c.c. is distributed in $\frac{1}{2}$ -oz. screw-cap Bijou bottles. These are steamed for one hour and then the bottles are filled further with the mixture, to ensure that when the swab is subsequently inserted they will be quite full. The caps are screwed down firmly and the bottles are kept for at least twenty-four hours, if they are then clear and colourless they are suitable for use. Absorbent cotton swabs on wooden applicators are contained in plugged swab tubes, all sterilised. (It is advisable to soak the swabs in a 1 per cent. suspension of charcoal e.g. 'Blood charcoal (B.D.H.)' and then sterilise them when dry, as this overcomes the inhibitory action of certain batches of agar. The material to be cultivated (pus or serous exudate) is taken on the swab, which is immediately plunged into the medium to about three-quarters of its depth; with scissors the top of the wooden applicator is then cut off flush with the medium and the cap screwed down firmly.

The complement-fixation test, as described earlier, may be applied in suspected cases in which the gonococcus cannot be demonstrated by direct methods.

GRAM-NEGATIVE ANAEROBIC MICROCOCCI (*VEILLONELLA*)

Members of this group have been described under different names, e.g. *Staphylococcus parvulus*, *Micrococcus parvulus* (see Veillon and Zuber Prévôt), and their taxonomic position has not been clearly defined, their association with the genus *Neisseria* is open to question, but the recognition of a genus under the designation *Veillonella* seems justifiable (see Bergey).

These organisms occur in the form of very small Gram-negative non-motile cocci about 0.3 to 0.4 μ in diameter, their general arrangement is in groups or masses, though short chains may be seen. Their optimum temperature for growth is 37° C. on ordinary media under strictly anaerobic conditions. Surface colonies are small and transparent, not usually exceeding 2 mm. in diameter when fully grown. The species designated *Veillonella parvula* is weakly hemolytic on blood agar and ferments glucose with acid and gas production and forms acid from certain other sugars, e.g. galactose and saccharose. It does not liquefy gelatin, but in culture media decomposes

polypeptides, producing gases such as hydrogen, carbon dioxide, and hydrogen sulphide, cultures having a somewhat fœtid odour ; indole is formed and nitrates are reduced to nitrites. Another species designated *V. gazogenes* differs from the former in being non-hæmolytic, non-saccharolytic and failing to

Hall and Howitt).

the mouth and alimentary tract
isolated from the appendix and

has been found in cases of pulmonary gangrene. While it has been isolated from suppurative lesions in connection with the mouth and alimentary tract and may occur as a secondary infecting organism, its pathogenicity has not been established.

CHAPTER VII

THE DIPHTHERIA BACILLUS AND ALLIED ORGANISMS (CORYNEBACTERIA)

THERE is no better example of the valuable contributions of bacteriology to scientific medicine than that afforded in the case of diphtheria. Not only did research supply means of identifying the causal organism and ascertaining its virulence, but the study of the toxins of the diphtheria bacillus explained the mode of production of the pathological changes and characteristic symptoms of the disease, and led to the discovery of specific methods of prophylaxis and treatment.

Bretonneau, from clinical and pathological observations, distinguished diphtheria as a specific disease, but the proof of this was effected by the discovery of the causal organism. The first account of the diphtheria bacillus was given by Klebs in 1883, who described its characters in the false membrane. It was first cultivated by Löffler from a number of cases of diphtheria, his observations being published in 1881, and to him we owe the first account of its characters in cultures and some of its pathogenic effects on animals. The organism has, for these reasons, often been spoken of as the Klebs-Löffler bacillus. By experimental inoculation with cultures, Löffler was able to produce false membrane on damaged mucous surfaces, but he hesitated to conclude definitely that this organism was the cause of the disease, because he did not find it in all the cases of diphtheria examined, also he was not able to produce paralytic phenomena in animals by its injection, and, further, he obtained the same organism from the throat of a healthy child. The organism became the subject of much inquiry, but its relationship to the disease was definitely established by the researches of Roux and Yersin, who showed that the most important features of the disease could be produced by means of the separated toxin of the organism. This was the original discovery of a bacterial exotoxin. Their experiments were published in 1888-90.

Without describing in detail the pathological changes in diphtheria, mention will be made of the outstanding features which require to be considered in connection with its bacteriology. At the site of infection, which is usually in the upper respiratory passages, there is a local inflammatory change with fibrinous exudation and cellular necrosis. In addition to this formation of false membrane, which may prove fatal by mechanical effects, the chief clinical phenomena are the symptoms of general poisoning, great muscular weakness, tendency to syncope, and albuminuria, also the marked paralysis which occurs later in the disease, and which may affect the muscles of the pharynx, larynx, and eye, or less frequently the lower limbs (being sometimes of paraplegic type), all these being grouped together under the term 'post-diphtheritic paralysis'. These effects are due to the diffused action on the tissues of the soluble toxins produced by the bacilli. The toxins act on the blood vessels and lead to general congestion of the organs, also oedema and a tendency to hæmorrhage. In the capillaries is often met with the kidney there is clotting of the secreting epithelium.

undergoing hyaline change followed by necrosis and associated with leucocytic infiltration—the auriculo-ventricular bundle may be involved; these lesions are important in relation to heart failure. Similar changes have been observed in cells of the central nervous system; and the peripheral nerves are also affected, as shown by disintegration of the medullary sheaths and swelling of axis cylinders. It should be stated here that all these conditions have been experimentally reproduced in animals by the diphtheria bacillus, or by its toxin. Other bacteria, however, are concerned in producing various secondary inflammatory complications in the region of the throat, such as ulceration, suppuration, and gangrenous change, which may be accompanied by symptoms of general septic poisoning. The detection of the bacillus in the false membrane or secretions of the throat is to be regarded as supplying the only certain means of diagnosis of diphtheritic infection, though it must be recognised that virulent or avirulent diphtheria bacilli may occur in the throat or nose unassociated with active or typical diphtheria, *e.g.* in carriers. On the other hand, fibrinous exudate may result from infection with other organisms, *e.g.* streptococci.

BACILLUS DIPHTHERIÆ (*CORYNEBACTERIUM DIPHTHERIÆ*)

MICROSCOPIC CHARACTERS. As seen in young cultures *B. diphtheriæ* occurs in the form of straight or, more frequently, slightly curved rods, which measure usually about 3 to 4 μ in length and 0.3 to 0.5 μ in thickness; both shorter and longer forms, however, are met with. The organism is non-motile and non-sporing. The individual bacilli vary in configuration, being rounded or tapered at their ends, and they stain unequally, either showing beading or giving a sort of barred marking (Fig. 31). Diphtheria bacilli frequently contain granules ('volutin' granules) which produce the beaded appearance (Fig. 32) and which with certain dyes give a metachromatic reaction, *e.g.* staining a purplish tint with polychrome methylene blue. The granules are stained a deep, bluish-black colour by Neisser's method, while the rest of the protoplasm is coloured



FIG. 31. Diphtheria bacilli from a twenty-four hours' culture on agar. Stained with methylene blue $\times 1,000$.

with the counterstain, *e.g.* chrysoidin. They are usually situated at the poles of the bacilli—'polar granules'—but occur in other parts of the substance, and the longer bacilli may contain several. It should be noted that these granules, while a pronounced morphological feature when the organism is growing on a serum medium, *e.g.* Löffler's, may not be developed on other media. The long form of diphtheria bacillus with well-marked granules is specially characteristic of the *mitis* type; the *gravis* type tends to show short forms in which granules may be absent or only irregularly present; as pointed out by Mair, barred staining is a feature of the *intermedius* type (*vide p.* 220). The ends of the bacilli are often expanded, especially in the longer forms, later these may form club-like structures which stain deeply, while the protoplasm becomes broken up into globules. Other bacilli may become thicker and segmented, and may even simulate a chain of cocci, and various stages of disintegration are seen (Fig. 33).

These aberrant forms, which are specially noticeable in cultures after several days' incubation, are usually regarded as the result of involution. Occasionally branched forms are met with. A characteristic feature in a film is the arrangement of the bacilli. They lie at various angles to one another, giving an appearance which has been compared to Chinese letters or cuneiform characters. This is apparently due to the snapping mode of division (p. 8), when a bacillus undergoes division the process at first occurs at one side and the two new individuals remain attached and set at an angle. It should be recognised that the size and general appearance vary with different strains of organisms and with different media, as well as with the duration of the growth. Sometimes quite short types are met with, rarely, in tryptic digest broth the culture may consist wholly of coccal forms arranged in clumps, diplococcal forms, and chains (Parish, 1927).

The bacilli are Gram-positive, though they are rather more easily decolorised than the pyogenic cocci. When decolorised, however, the granules may still retain the Gram's stain.

In films from the pharynx or from the membrane the bacilli have the same general characters, but they tend on the whole to stain more evenly and granules are not so pronounced (Fig. 36).

CULTURAL CHARACTERS The diphtheria bacillus grows best in culture at the temperature of the body, growth still takes place at 22°C , but practically ceases about 20°C , it occurs under both aerobic and anaerobic conditions. The best media are those which contain blood or serum, e.g. Löffler's coagulated serum medium, but growth occurs also on the ordinary agar media. If inoculations are made on the surface of coagulated serum with a piece of diphtheria membrane, colonies of the bacillus appear in twelve hours, and are well formed within twenty-four hours. The colonies are small circular disks of opaque whitish colour, the centre being thicker and of

darker greyish appearance than the periphery. Their margins are at first regular, but later they become wavy or even crenated. On the second or third day they may reach 3 mm in size, but when numerous they remain smaller. Their surface becomes distinctly granular, and when there is a

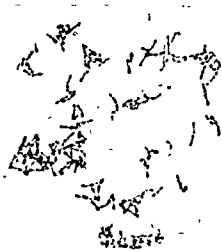
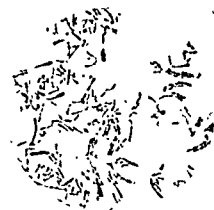


FIG. 32 Diphtheria bacilli, showing beaded appearance due to deeply stained granules from a twenty-four hours' culture on Löffler's serum. Stained by Neisser's method $\times 1,000$.



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mass of growth it comes to have a yellowish tint. On *ordinary agar* the colonies have much the same appearance, but grow less quickly, and some-

have been distinguished (Cowan). In *gelatin* at 22° C. a stab culture shows a line of small white colonies along the track of the wire, while the growth at the surface is only slight. In none of the media does any liquefaction occur. In *broth* the character of growth varies with the biological type (*vide infra*)—either a uniform turbidity is produced or the growth is granular, forming a deposit and a powdery layer on the wall of the vessel; in this case if the growth is started on the surface and the container is kept at rest, a distinct scum may form, which is especially suitable for the development of toxin (*vide infra*).

Growth factors β -alanine, pimelic, and nicotinic acids have been found to act as

however, appears to be an additional growth factor for certain *gravis* strains, while other strains themselves synthesise this factor (Evans *et al.*) It has also been pointed out that some *mitis* strains are inhibited by heated blood due in part to the hæmatin present, this effect being counteracted by reducing substances (Glass).

In recent years for the diagnostic cultivation of the diphtheria bacillus extensive use has been made of *agar media containing potassium tellurite and heated or laked blood* as recommended by McLeod and others. Tellurite renders these media highly selective for the diphtheria bacillus and allied diphtheroid organisms, since suitable concentrations which permit their growth, inhibit the growth of other bacteria present in the mouth and throat, e.g. staphylococci, streptococci, pneumococcus, *D. catarrhalis*, etc. the tellurite is also reduced by the diphtheria and diphtheroid bacilli and their colonies assume a black, greyish-black, or slate-grey coloration. The morphology of colonies is also more characteristic and, as will be shown, on many such media different types of the diphtheria bacillus can be distinguished by colony appearances. Rabbit blood is specially suitable for this purpose. On such media, however, the characteristic metachromatic granules demonstrable by Neisser's staining method are not observed or only imperfectly seen.

Further reference is made below to the cultivation of the diphtheria bacillus in relation to toxin production.

Types Three biological types of the diphtheria bacillus, designated respectively *gravis*, *mitis*, and *intermedius* have been distinguished by Anderson, Happold, McLeod and Thomson (Leeds), chiefly according to cultural characters on a medium containing blood and tellurite; and they originally related these to different clinical types of the disease. *B. diphtheriæ gravis* (so called because of its association with a severe type of diphtheria) grows in the form of flattened, lustreless colonies of irregular outline, which often exhibit a 'daisy head' formation; it exhibits a granular growth and pellicle in broth. *B. diphtheriæ mitis* (usually associated with a milder type of disease) has a smooth, convex colony which is partly translucent, it produces a uniform turbidity in broth. The *intermedius* form yields a small lustreless colony with a domed centre and flat irregular margin. The Park Williams No. 8 strain, which has been extensively used for toxin production, is of this nature. On heated (rabbit) blood agar reinforced with

rabbit serum the colonies of the *intermedius* type have been described as exhibiting a characteristic olive-green colour (Gordon and Higginbottom). The question of the significance of the biological types of the diphtheria bacillus will be discussed later

BIOCHEMICAL REACTIONS. The action of *B. diphtheriae* in fermentation tests has been extensively studied and is of considerable importance. It may be said that the organism produces acid without gas formation from glucose, maltose, and galactose, and no acid from lactose, saccharose, and mannitol. Glucose and saccharose are the sugars employed for practical purposes. In carrying out the tests it is important to ensure that there is abundant growth in the medium, and for this reason a liquid serum medium such as Hiss's, with 1 per cent of a suitable peptone added is to be recommended, phenol red being used as indicator and the pH adjusted to 7.2. In Hiss's medium the development of acid is attended by the formation of clot. The fermentation of starch and glycogen is a characteristic feature of the *gravis* form of the diphtheria bacillus, whereas the *mitis* and *intermedius* forms have no action

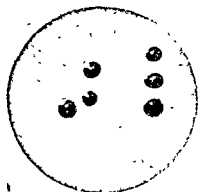


FIG. 34. Colonies of *B. diphtheriae mitis* on McLeod's medium (48 hours' growth) $\times 1\frac{1}{2}$



FIG. 35. Colonies of *B. diphtheriae gravis* on McLeod's medium (48 hours' growth). $\times 1\frac{1}{2}$

on these polysaccharides. Reference to the fermentative properties of allied organisms is made below.

Hæmolysis. The fact that certain strains of *B. diphtheriae* may produce hæmolysis when grown on a blood medium has long been recognised. It has also been found that lysis may result when blood is added to fluid cultures. Whether this effect is due to a diffusible toxin similar to the hæmolysins of certain other bacteria, e.g. streptococci, remains doubtful. It may be noted that while the *mitis* type is hæmolytic to ox and rabbit erythrocytes the *gravis* form lacks the property of lysing ox blood though it is hæmolytic to rabbit corpuscles. The *intermedius* type is non-hæmolytic to both ox and rabbit corpuscles.

VIABILITY. In cultures the bacilli possess long duration of life; at room temperature they may survive for two months or longer. In the moist condition, whether in cultures or in membranes, they have a low power of resistance to heat, being killed at 60° C. in a few minutes. On the other hand, in the dry condition they have great resisting powers. In membrane which is perfectly dry, for example, they can resist a temperature of 98° C. for an hour. Dried diphtheria membrane, kept in the absence of light and at the room temperature, has been proved to contain diphtheria bacilli still living and virulent at the end of several months. The presence of light and moisture causes them to die out more rapidly. Corresponding results have

been obtained with bacilli taken from cultures and kept on dried threads. It has been shown that the diphtheria bacillus may survive in the floor dust of fever hospital wards with resultant contamination of the air (Crosbie).

Distribution of the Bacillus. *B. diphtheriae* may be found in the membrane wherever it is formed, and also occurs in the secretions of the pharynx and larynx in the disease. Virulent diphtheria bacilli have been found in a considerable proportion of cases of fibrinous rhinitis. In the case of any nasal lesion, however, the test for virulence should always be made, as 'diphtheria-like' bacilli without virulence are of comparatively common occurrence.

In diphtheria the membrane has a somewhat different structure according as it is formed on a surface covered with stratified squamous epithelium, as in the pharynx, or on a surface covered by ciliated epithelium, as in the trachea. In the former situation

necrosis of the epithelium occurs either uniformly or in patches, and along with this there is marked inflammatory reaction in the connective tissue beneath, attended by abundant fibrinous exudation. The necrosed epithelium becomes raised up by the fibrin, and its interstices are also filled by it; fibrinous exudation also occurs around the vessels in the tissue beneath. The membrane is thus firmly adherent, and when artificially detached it leaves a bleeding surface. In the trachea, on the other hand, the epithelial cells rapidly become shed, and the membrane is found to consist almost exclusively of fibrin with leucocytes, the former arranged in a reticulated or somewhat laminated manner, and varying in density in different parts. The membrane lies upon the basement membrane, and is comparatively loosely attached.

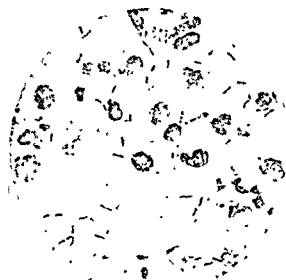


FIG. 36. Film preparation from diphtheria membrane, showing numerous diphtheria bacilli. One or two degenerate forms are seen near the centre of the field. (Cultures made from the same piece of membrane showed the organism to be present in practically pure condition.) Stained with methylene blue $\times 1,000$.

The position of the diphtheria bacilli varies somewhat in different cases, but they are most frequently found lying in

clumps in the spaces between the fibrin, towards the superficial, that is, usually, the oldest part of the false membrane (Fig. 37). There they may be in a practically pure condition, though streptococci and occasionally some other organisms may be present along with them. They may occur also in deeper parts, but are rarely found in the fibrin around the blood vessels. On the surface of the membrane they may be also seen lying in large numbers, but are there accompanied by numerous other organisms. Occasionally a few bacilli have been detected in the lymphatic glands. As Löffler first described, they may be found after death in pneumonic patches in the lung, these being due to a secondary extension by the air passages. They have also been occasionally found in the spleen, liver, and other organs after death. Such occurrences are probably to be explained in most cases by an entrance into the blood stream shortly before death. In a few instances they have been cultivated from the blood during life. Under exceptional conditions the organism may spread from the original site of infection and by the blood stream reach other parts of the body, localising there and producing active lesions. Thus ulcerative

endocarditis due to the diphtheria bacillus has been reported (see Graetz), also meningitis (Kalbfleisch and Kretschmer) and cases of bacilluria (Lanza). It may be accepted, however, that the growth of the organism is mainly local, that it does not invade the tissues to any extent, and that its important effects are produced by toxins absorbed from the site of growth. The diphtheria bacillus may also infect other mucous membranes, thus diphtheria of the conjunctiva has been found occasionally, and similar affections of the vulva and vagina may occur. In most of the recorded cases of such lesions, however, the identity of the bacilli has not been fully established, some of them are described as responding to treatment with antitoxin. During the war of 1911-18 virulent diphtheria bacilli were cultivated from wounds in a small proportion of cases, their presence was not attended by any special effects. Cutaneous diphtheria may sometimes occur and takes the form of ulcers with fibrinous exudate or of vesicular or pustular lesions, diphtheritic paronychia may also be met with. Organisms with all the biological characters of the diphtheria bacillus have been isolated from cutaneous sores in tropical and sub-tropical countries but have usually proved to be non-virulent for guinea-pigs and non-toxicogenic. Their relationship to the diphtheria bacillus remains doubtful. True diphtheria bacilli have also been isolated from such sores but may represent a secondary infection.



FIG. 100

$\times 1,000$

Association with other organisms. In diphtheria the pyogenic organisms—streptococci, staphylococci, pneumococcus, etc.—are practically always present in the pharynx, occurring there in varying numbers and combinations. Haemolytic streptococci are, however, relatively abundant in certain cases and can hardly be without effect in aggravating the condition. They are often found lying side by side with the diphtheria bacilli in the membrane, and also penetrating more deeply into the tissues. In some cases of tracheal diphtheria streptococci have been found alone at a lower level in the trachea than the diphtheria bacilli, where the membrane is thinner and softer, the appearance in these cases being as if the streptococcal infection were spreading more rapidly than the diphtheritic condition. Further, some of the complications of diphtheria are due to the action of pyogenic organisms. The extensive swelling of the tissues of the neck, sometimes attended by suppuration in the glands and also various hemorrhagic conditions, have been found to be associated with their presence. The diphtheritic lesion enables them to get a foothold in the tissues, where they exert their usual action and may lead to extensive suppurative change or even to septicæmia. In cases where a gangrenous process is superadded, a great variety of organisms may be present, some of them being anaerobic.

Effects of Experimental Inoculation. In considering the effects produced in animals by experimental inoculation of pure cultures, we have to keep in view the local changes which occur in diphtheria, and also the symptoms of general poisoning.

As Löffler stated in his original paper, inoculation of the healthy mucous membranes of various animals with pure cultures causes no lesion, but the formation of false membrane may result when the surface is injured by scarification or otherwise. A similar result may be obtained when the trachea is inoculated after tracheotomy has been performed. The membrane produced in such experiments is usually less firm than in human diphtheria, and the bacilli in the membrane are less numerous. Rabbits inoculated after tracheotomy often die, and Roux and Yersin were the first to observe that in some cases paralysis might appear before death.

Subcutaneous injection in guinea-pigs of diphtheria bacilli in a suitable dose produces death within thirty-six hours to four days. At the site of inoculation there is usually an area of inflammation with some necrosis, whilst in the tissues around there is extensive inflammatory œdema, often associated with hæmorrhages, and there is also swelling of the corresponding lymphatic glands. The internal organs show general congestion, the suprarenal glands being especially enlarged and congested and often hæmorrhagic. The renal epithelium may show cloudy swelling, and there is often effusion into the pleural cavities. After injection the bacilli increase in number for a few hours, but multiplication soon ceases, and at the time of death they may even be less numerous than when injected. The bacilli remain practically local, cultures made from the blood and internal organs usually giving negative results. If a non-fatal dose of a culture be injected, a local necrosis of the skin and subcutaneous tissue may follow at the site of inoculation.

In rabbits, after subcutaneous inoculation, results of the same nature follow, but these animals are less susceptible than guinea-pigs, and the dose requires to be proportionately larger. Roux and Yersin found that after intravenous injection the bacilli rapidly disappeared from the blood, and when 1 c.c. of a broth culture had been injected no trace of the organisms could be detected by culture after twenty-four hours, nevertheless the animals died with symptoms of general toxæmia, nephritis also being often present. The dog and sheep are also susceptible to inoculation with virulent bacilli, but the mouse and rat enjoy a high degree of immunity.

Intracutaneous injection has been found to be of service in testing the virulence and thus in the identification of the diphtheria bacillus, especially when used in conjunction with the injection of antitoxin. Injection with a fine needle of a small amount of a culture of a guinea-pig produces a circumscribed superficial necrosis in from one to two days. Received previously an injection of, say, 250 units of antitoxin, the result is negative. The result is also negative (without antitoxin) in the case of an avirulent strain. This test can also be carried out on the rabbit.

Toxin. In the above experiments the symptoms of poisoning, and ultimately a fatal result, occur when the bacilli are not increasing in number, or even after they have practically disappeared. Accordingly, Roux and Yersin inferred that the chief effects were produced by a toxin, and this supposition they proved by showing that cultures of the bacilli, after three or four weeks'

The filtrate when injected into guinea-pigs and other animals produced practically the same effects as the living bacilli; locally there is little fibrinous

exudation but a considerable amount of inflammatory cedema, and, if the animal survives long enough, necrosis of the superficial tissues in varying degree may follow. The toxicity may be such that 0.001 to 0.00025 c.c. is fatal to a guinea-pig in five days. One point of much interest is the high degree of resistance to the toxin possessed by mice and rats. Roux and Yersin, for example, found that 2 c.c. of toxin, which was sufficient to kill a rabbit in sixty hours, had no effect on a mouse, whilst of this toxin even $\frac{1}{15}$ c.c. produced extensive necrosis of the skin of the guinea-pig.

After injection either of the toxin or of the living bacilli, when the animals survive long enough, a state of marasmus sets in or paralytic phenomena occasionally occur. In the latter condition the hind-limbs are usually affected first, the paralysis afterwards extending to other parts, though sometimes the fore-limbs and neck first show the condition. Some times symptoms of paralysis do not appear till two or three weeks after inoculation. After paralysis has appeared, a fatal result usually follows in the smaller animals, but in dogs recovery may take place. It is to be noted in this connection that paralytic symptoms may occur in the human subject even after treatment with antitoxin. The production of these paralytic phenomena was ascribed by Ehrlich to 'toxone', as they occurred specially when there was injected along with the toxin sufficient antitoxin to neutralise the more rapidly acting toxin proper. This toxone was supposed to have a different 'toxophore' group from that of the ordinary toxin, so that it produced the late nervous phenomena, while its local action on the tissues was very slight. Owing to the weaker affinity for antitoxin which he postulated, much of it might be left un-neutralised. Prigge's work has indicated, however, that toxone has no separate existence as a constituent of diphtheria toxin, since he was able to reproduce the paralytic effects regularly in guinea-pigs by repeated injections of minute doses of diphtheria toxin—without any admixture of antitoxin—over a long period. Thus paralysis is the result of a *chronic* intoxication with the products of the diphtheria bacillus.

Preparation of the toxin. The obtaining of a very active toxin in large quantities is an essential in the preparation of anti-diphtheritic serum. Certain conditions favour the development of a high degree of toxicity in broth cultures: namely, a free supply of oxygen, the presence of a large proportion of peptone or products of tryptic digestion, the absence of substances which produce an acid reaction, and an initial reaction of pH 7.5 to 8. Recently it has been shown that there is a narrow range of content in iron which is critical for the abundant production of this toxin (Lappenhimer and Johnson). In the earlier work a current of sterile air was made to pass over the surface of the medium, as it was found that by this means the period of acid reaction was shortened and the toxin formation favoured. This expedient was later considered unnecessary if an alkaline medium free from glucose was used and the cultures were made in shallow layers of medium. It was recognised by the early workers that the occurrence of a surface pellicle of growth favoured toxin production and this has been emphasised by Pope and Healey. The absence of glucose may be attained by fermenting the meat infusion with yeast. Martin employed a medium composed of equal parts of freshly prepared peptone and glucose-free veal broth and obtained with it good yields of toxin. As a constituent of medium for toxin production commercial peptones vary considerably and a specially prepared tryptic digest provides the most satisfactory basis. A medium prepared from a tryptic digest of horse flesh which has been much used is Hartley's broth. Certain organic substances have been found to be valuable in augmenting the formation of toxin, e.g. sodium acetate, sodium lactate and maltose (Pope and Healey). It may be noted that maltose is only slowly converted to glucose by the growth of the organism and its presence does not lead to excessive acid production. A medium fulfilling these requirements is that of Pope and Smith. It incorporates a glucose-free meat infusion, a tryptic digest of meat, 0.3 per cent maltose and 0.1 per cent sodium lactate. Such culture media apparently contain factors valuable for toxin production which are heat-labile, and therefore it is

advisable to sterilise by filtration through a Seitz filter followed by ten minutes steaming at 100° C. As emphasised above, the reaction of the media should be accurately adjusted within the limits mentioned (pH 7.5 to 8). Incubation is carried out at 33° to 35° C. There is in all cases a period at which the toxicity reaches a maximum; by recent methods the greatest toxicity is reached about the tenth day or even earlier. An important factor, as mentioned above, is the oxygen tension which depends on the relative area of surface of the medium exposed to the air, thus toxin formation in layers of medium, e.g. has been reached the c

of toluol; it is then shaken on several occasions for two or three days. Filtration through a Seitz filter yields a bacteria-free filtrate which represents the crude toxin. When toxin is to be used for testing purposes it is kept in an ice-chest for about twelve months, till it has been 'ripened' or stabilised. It may be added that the power of toxin formation varies much in different strains of the diphtheria bacillus. A strain designated Park-Williams No. 8 has been extensively used for the production of diphtheria toxin and has proved most suitable for the purpose.

Though the media described above have been much used for toxin production it has been found possible to obtain highly potent toxin in a synthetic medium containing amino-acids, maltose, inorganic salts, and certain growth factors, such as pimelic and nicotinic acids.

Properties and nature of the toxin. The toxic substance in filtered cultures is a relatively unstable body. When kept in sealed tubes in the absence of light, it may preserve its powers little altered for several months, but, on the other hand, it gradually loses them when exposed to the action of light and air. As has been shown, the toxin probably does not become destroyed, but its 'toxophore' group undergoes deterioration, so that *toxoid* is formed which has still the power of combining with antitoxin and of stimulating the production of antitoxin *in vivo*. Heating at 58° C. for two hours destroys the toxic properties in great part, but not altogether. When, however, the preparation is evaporated to dryness, the toxin has much greater resistance to heat. One striking fact, discovered by Roux and Yersin, is that after an organic acid, such as tartaric acid, is added to the toxin the toxic property disappears, but it can be in great part restored by again making the fluid alkaline. Reference has been made in Chapter II to the protein nature of the toxin, which has been isolated in purified state from cultures in synthetic medium. Its molecular weight has been estimated at 15,000 to 72,000 and the lethal dose for a guinea-pig (of 250 grms weight) is 0.0001 mgm (Pappenheimer).

Toxic bodies have also been obtained from the tissues of those who have died from diphtheria. Roux and Yersin, by using a filtered watery extract from the spleen in very virulent cases of diphtheria, produced in animals death after wasting and paralysis, and also obtained similar results by employing the urine.

Variations in Virulence and in Toxin Production. The distinction between virulence—the power to invade the tissues and produce disease—and the faculty of toxin production by cultures *in vitro* has already been emphasised (p. 54), it is well illustrated in the case of *B. diphtheriae*. For comparative tests of virulence known quantities of bacilli from a young culture on solid medium should be used for injecting a guinea-pig, as in fluid cultures the presence of variable amounts of toxin modifies the result. As shown by the amount of culture necessary to produce death of a guinea-pig, it may be said that all degrees of virulence are met with among strains freshly cultivated from cases of diphtheria and carriers; and non-virulent strains may be found in carriers. The virulence of a given strain, moreover, is usually well maintained in culture. A certain falling off has sometimes been observed after a time. Further, unlike what obtains with other organisms, little or no increase

of virulence can be produced by means of *passage*. It is now generally accepted that virulence is a relatively stable property. In the usual subcutaneous test for virulence a whole growth on a serum slope culture is used, and either death of the animal or no pathogenic effect results. Some non-virulent strains have been found, however, to produce a small amount of toxin in media or to give rise to a small amount of antitoxin on injection. These facts indicate that all gradations occur, and suggest that the non-virulent organisms belong to the same species as the virulent.

A certain correspondence exists between virulence and toxin production, inasmuch as every virulent strain produces toxin, but the two properties do not run in a parallel manner, as some strains of moderate virulence are very active in toxin production. As has been mentioned earlier, in the preparation of antitoxin the obtaining of a very powerful toxin is necessary, and for this purpose many strains may have to be tested before a satisfactory toxin producer is obtained. The strain obtained by Park and Williams many years ago, known as No. 8, has been used very extensively all over the world and is noted for its properties in this respect. This is an illustration of the well-recognised fact that the power of toxin production usually remains unchanged for a long period in conditions of artificial cultivation; this strain, however, is not highly virulent.

Cultures derived originally from a single diphtheria bacillus have yielded both virulent and non-virulent strains, and Cowan has isolated from a virulent culture, by repeated plating, colonies of 'rough' type consisting of organisms which were non-virulent and did not produce toxin. Also the observations made by Okell on virulent and avirulent strains isolated from the same patient and from different patients in the same epidemic suggest that *in vivo* virulent bacilli may occasionally yield non-virulent variants. This may explain the finding of non-virulent strains in carriers, but the original idea of Roux and Yersin that after an attack of diphtheria the bacilli in the throat gradually became attenuated and ultimately non-virulent has not been substantiated. It should be noted that the transformation of a non-virulent into a virulent strain has not been observed. It has been stated that the diphtheria bacilli recovered from cases in which antitoxin treatment has failed, are characterised by their high virulence.

The significance of the biological types of the diphtheria bacillus When the *gravis* and *mitis* types were first distinguished it was pointed out that a high correlation existed between the clinical severity of the disease and the cultural type, the *gravis* form being associated with cases of diphtheria of unusual severity, which exhibited a relative refractoriness to antitoxin treatment. The Leeds workers found that 95 per cent. of strains belonged to these types, though a small percentage were classified as 'intermediate'. Later studies have all shown that this *intermedius* form occupies a very prominent position among the three types (Robinson and Marshall, Wright and Christison, Carter, Murray, Cooper *et al*). The main characters on which differentiation has been based are the colony features (*vide p. 220*) and the fermentation of polysaccharides, e.g. starch, the *gravis* type fermenting this substance, while the *mitis* and *intermedius* are inactive. It is unnecessary here to refer to the various other differential features, but it has been emphasised that in Great Britain virulence for animals is a very constant character of the *gravis* strains, the *intermedius* are virulent with few exceptions, the *mitis* strains are usually virulent but vary in this respect. In addition to the original types, Wright and Christison have described three others all exhibiting a *gravis* form of colony but differentiated according to starch fermentation and virulence, and suggest a numerical denomination of types.

type plays no part in the spread of the disease, and that individuals harbouring it are not a danger to the community. Moss, Guthrie and Marshall have found that the carrier state (without pathogenic effects) is easily produced by inoculating the throat of healthy persons with non-virulent diphtheria bacilli. Prior administration of antitoxin did not prevent the development of the bacilli in the throat. The carrier state so produced tended to persist and there was no evidence of any change in the characters of the organisms, which remained non-virulent. Further, no cases of clinical diphtheria occurred among the associates of such carriers. These workers have also recorded experiments in which Schick-positive and -negative persons (*vide infra*) were inoculated with a virulent culture of the diphtheria bacillus, typical clinical diphtheria resulted in the positive reactors, whereas the negative reactors became carriers without showing clinical effects. As a rule, chronic carriers give a negative Schick reaction, that is, are immune. In the investigation of healthy contact carriers the Schick test accordingly adds valuable information when a suspicious organism has been cultivated from the fauces. Thus, if the suspected carrier is Schick-positive, *i.e.* non-immune, the organism is likely to be non-virulent.

In one or two exceptional instances diphtheria bacilli have been obtained from the lower animals, *e.g.* cow and horse, but there is no evidence that animal infection plays any part in the spread of the human disease. It may also be added that the so-called 'diphtheritic' diseases of animals are due to quite different organisms.

Immunity. This has been discussed in the general chapter on Immunity. It is sufficient to state here that, as first shown by Pasteur and Chamberland, immunity, against both the bacilli and their separated toxin (or toxoid). As a result of the immunisation, antitoxin appears in the serum, and this is capable of protecting animals either against infection with diphtheria bacilli or against their toxin. It also has a curative effect in animals which are already the subjects either of infection or intoxication. The production and standardisation of diphtheria antitoxin for medical use are dealt with in Chapter III.

Reference has been made (p. 83) to the fact that in a certain proportion of horses in the natural state antitoxin is present in the blood, and a similar statement also holds with regard to the human subject. The presence of the antitoxin is shown by mixing a certain amount of the serum with a small quantity of toxin and injecting some of the mixture intracutaneously in the guinea-pig (Römer's test), an injection of the same amount of toxin alone is made at another spot as a control. The application of this test gave important knowledge with regard to the presence of antitoxin at different ages, but for practical purposes it has been superseded by the Schick test, which may now be described. The presence of $\frac{1}{10}$ to $\frac{1}{100}$ unit of antitoxin per c.c. of serum is probably sufficient to afford the individual protection against infection.

SCHICK TEST. This test depends upon the fact that when a minute amount of diphtheria toxin is introduced into the skin an inflammatory reaction results, provided that insufficient antitoxin is present in the blood to neutralise the effects. As has just been stated, however, the blood of a certain number of individuals contains a small though sufficient amount of antitoxin, and in these the reaction does not occur—the result is negative. The presence of antitoxin in the blood is attended by a certain degree of immunity, and thus the test gives an indication of the presence or absence of immunity, according as the result is negative or positive respectively. The

amount of toxin injected is of importance ; it should be sufficient to ensure a positive reaction in an individual without antitoxin, and it should not be in excess of the amount neutralised by the antitoxin supposed to be associated with immunity.¹ The dose of toxin used is that amount which is just neutralised by $\frac{1}{1000}$ unit of antitoxin, as tested by intracutaneous injection in a normal guinea-pig. For the purpose an old toxin preparation is usually selected in which some of the toxin has undergone alteration to toxoid, and this is diluted with a buffer solution² so that 0.2 c.c. of the dilution contains the test-dose. This buffered preparation can be kept for some time without deterioration. (The 'test-dose' of toxin must contain sufficient toxin to ensure that when $\frac{1}{2}$ of this amount is injected intracutaneously into a normal guinea-pig a definite local inflammatory reaction results, while $\frac{1}{10}$ of the test-dose must not cause such a reaction) Occasionally there occur pseudo-reactions due to supersensitiveness or allergy towards certain substances other than the toxin in the filtrate, this allergy being met with alike in immune and non-immune subjects ; however, a marked pseudo-reaction is usually associated with a relatively high content of antitoxin in the blood. Diphtheria toxin is readily destroyed by heating, whereas the substances leading to pseudo-reactions are not. A similar preparation containing the same concentration of toxin heated to 70° C. or higher for at least five minutes can thus be used as a control, and of course any effect produced by it will be of the nature of a pseudo-reaction, that is, will not be due to the true toxin. Test and control preparations for carrying out this test are now supplied commercially.

The test is made by intracutaneous injection on the flexor aspect of the forearm, one arm being used for the toxin, the other for the heated toxin as a control. In the case of a positive reaction (in a non-immune individual without pseudo-reaction) an area of redness and slight swelling appears after twenty-four to forty-eight hours, and reaches its maximum about the fourth day, when it may measure up to 2 cm. or more in diameter. It persists for seven days and then gradually fades, becoming brownish in tint, and there is usually some desquamation of the surface epithelium. The control injection on the other arm is, of course, attended by no result. If the reaction is negative, no change follows at the site of injection of the diluted toxin, the condition of the two arms being similar.

A pseudo-reaction is indicated also by an area of redness, but it appears earlier, often after six hours, is less intense and less definite at its margins than the positive

If it is associated

on the two arms

unheated toxin will be more marked and persist longer than the reaction on the control arm. The difference is, as a rule, specially marked on the fourth to seventh day, when the reading should be taken ; a positive reaction is then still present, while a pseudo-reaction has completely or almost completely faded. Pseudo-reactions are most frequently met with in persons over six years of age, and in them a control is absolutely necessary.

Extensive series of observations at different periods of life have been carried out by the Romer and by the Schick method, and the results correspond on the whole fairly closely. It may be said that, at the time of birth, the serum of 80 per cent. of children contains antitoxin, and thus they give

¹ The *Therapeutic Substances Regulations* prescribe the standards of potency of the test-dose of toxin to be used in the test.

² The solution contains borax, boric acid and sodium chloride with Witte's peptone or human serum as a stabiliser.

a negative Schick reaction, that this proportion falls for a year or two, at first rapidly, and that then there is a gradual rise until in adult life the original level is reached. The age of maximum susceptibility may be said to be from ten months to six years. It is generally agreed that the presence of antitoxin in the infant represents a passive immunity transferred from the mother, hence the percentages of mothers and of infants whose serum contains antitoxin correspond. It is of interest that such passive immunity persists for several months after birth, as compared with the very short duration of passive immunity produced by injecting an antitoxic serum from an animal. In the former case the antibody is a homologous protein; in the latter heterologous, and therefore more rapidly eliminated from the system. The gradual diminution of the number of Schick-positive reactors after the sixth year is supposed to be due to a process of active immunisation occurring as the result of the individuals harbouring diphtheria bacilli and of slight infections by the organism. This, however, may not be the complete explanation, since observations on isolated communities have indicated that although diphtheria infection is absent, the proportion of Schick-negative reactions increases with age much as in the large communities in which diphtheria is a prevalent disease. Accordingly the possibility must be considered that there is a natural or physiological development of diphtheria antitoxin as is the case with certain other 'natural' antibodies. It is true, however, that as a general rule the percentage of Schick-positive reactors is greater in rural than urban communities and in children of the higher social classes than among those who lead a less 'sheltered' life and are more exposed to herd infection.

PROPHYLACTIC IMMUNISATION. This was originally carried out by active immunisation with a small amount of toxin partly neutralised by antitoxin, the amount of the mixture used for injection (e.g. 1 c.c.) being only slightly toxic to a guinea-pig. The method was applied on a large scale by Park, Zingher, and others, and the results proved distinctly favourable. Three consecutive injections were given with an interval of a week between, and the immunisation was recommended to be carried out in young children from about a year old, so as to give protection during the highly susceptible period of life. Immunity, as shown by a previously positive Schick reaction becoming negative, developed somewhat slowly, but in 90 per cent. of the cases the reaction became negative after three months. Observations were originally carried out on 200,000 school children in America; half of these were tested for the Schick reaction, and those giving a positive reaction were immunised, the other half were used as controls. The result was that amongst the controls the number of cases of diphtheria which occurred was five times as great as amongst those tested and treated. Further, all the severe cases occurred amongst the controls (see Park and Williams).

It must be noted in connection with the Schick test as a measure of immunity that the reactivity of the skin of different individuals to the toxin may show considerable variation. Accordingly, among persons who have received any form of diphtheria prophylactic, one may react negatively although his serum contains less antitoxin than that of another who still reacts positively.

Immunisation with toxoid. More recently for purposes of active immunisation use has been made of the toxoid introduced by Ramon, also called anatoxin, which results from the action of formalin on diphtheria toxin. Toxin is converted to toxoid by the addition of 0.3 per cent. formalin and keeping the preparation for three weeks at 37° C. The final product is controlled by injecting 5 c.c. subcutaneously into a guinea-pig and ascertaining in this way that it is non-toxic. Glenny and his co-workers have found,

however, that on account of its rapid elimination, toxoid does not provide a continuous stimulus, but when it is converted into a relatively insoluble precipitate a more effective antigenic action is secured. This may be done by precipitation of the toxoid with potash alum, a similar effect is obtained by using the floccules which result when toxoid is mixed with antitoxin.

Various preparations of toxoid have been applied practically for prophylaxis: (1) the original formol-toxoid (F.T.), (2) alum-precipitated toxoid made up in the form of a suspension (A.P.T.); (3) toxoid-antitoxin mixture (T.A.M.) containing toxoid partially neutralised by antitoxin, and (4) toxoid-antitoxin floccules (T.A.F.), a suspension of the flocculent precipitate produced when toxoid and antitoxin are mixed in neutralising proportions.¹ Formol-toxoid and alum-toxoid have a tendency to cause troublesome local and general reactions when administered to older children (over six years of age) and adults. This can be predicted in the individual case by a preliminary test originally advocated by Moloney and Fraser—the 'Moloney test'—in which a minute amount of the toxoid is injected intradermally at the same time as the preliminary Schick test is carried out. The occurrence of a local skin reaction indicates that the individual is sensitive, and if so the dosage of the prophylactic should be reduced or an alternative preparation employed. Both the toxoid-antitoxin mixture and the toxoid-antitoxin floccules are much less likely to produce these reactions. Of these the latter (T.A.F.) is the more effective antigen and should in fact be used in preference to ordinary toxoid or alum-precipitated toxoid in older children and adults, especially if a preliminary test with toxoid reveals sensitiveness. This prophylactic is, however, more difficult to prepare and is somewhat more costly.

In recent years immunisation has usually been carried out with only two of these preparations—alum-precipitated toxoid for young children and toxoid-antitoxin floccules for older children and adults. Two doses of alum-precipitated toxoid (50 L₁ doses per c.c.) are usually given at an interval of four weeks, 0.1 c.c. (or 0.2 c.c.) and 0.5 c.c. of the standard preparations, in the case of toxoid-antitoxin floccules three doses should be administered at intervals of four weeks, in each case 1 c.c. of the standard preparations. The doses of these prophylactics are administered by intramuscular injection in the upper arm. The most suitable age for immunising children is from the eighth to the tenth month of life. Immunisation should be repeated when the child first enters school—a 'boosting' dose of 0.2 c.c. alum-precipitated toxoid.

by a
been
weeks
three to six months after immunisation, 4 per cent. gave a positive reaction after two years and 18 per cent. after six years (Duke and Stott). Recently, as a result of experimental and clinical observations, the use of a larger first dose of alum-precipitated toxoid in infancy has been recommended, viz. 0.5 c.c., followed by a similar amount after an interval of three to six months, also a dose of 0.2 c.c. in the third year as well as on beginning school (see Parish, 1947).

Active immunisation with toxoid has been found highly effective in controlling the incidence of diphtheria and more particularly the mortality from the disease. It does not appear that as a result of active immunisation a general increase of the carrier rate in the community is to be expected.

¹ *The Therapeutic Substances Regulations* prescribe tests which ensure that commercial supplies of 'Diphtheria Prophylactic' (toxin antitoxin, toxoid etc.) shall not be too toxic and at the same time shall possess efficient immunising properties.

THERAPEUTIC EFFECTS OF DIPHTHERIA ANTITOXIN. The use of antitoxin for the prevention and treatment of diphtheria constituted the first great contribution of bacteriology to practical therapeutics. Soon after its introduction in 1891 Roux and his co-workers published strong clinical evidence of the value of antitoxin. Fibiger in 1898 reported a series of cases over a period of a year, those admitted on alternate days being treated with and without antitoxin. Of two hundred and thirty-nine patients who received antitoxin eight died (3.3 per cent.), while of two hundred and forty-five who did not receive it thirty died (12.2 per cent.); statistically in favour of antitoxin treatment. The greater is the mortality from the is clinically suspect antitoxin should

be administered as soon as possible and without waiting until the bacteriological diagnosis is established. For treatment of the disease the practice as to method of administration and dosage still varies, but larger doses are now given than formerly, and it is generally recognised that the best routes of injection are the intramuscular and intravenous. The former is the more convenient, but the latter gives the more rapid distribution of antitoxin throughout the body and is thus more effective. Further, the longer treatment has been delayed or the severer the disease, the more is the intravenous method to be preferred and the larger should be the dose. Subcutaneous injection is much less efficient, as the absorption of antitoxin into the circulation takes place relatively slowly. Some authorities give the antitoxin in a single dose, others in two or more doses. Theoretically, there appears to be no advantage in the latter method, as a single dose will keep the antitoxin content of the blood during the disease at a level more than sufficient to neutralise the toxin, nevertheless, some maintain that administration in more than one dose has an advantage. The dose, of course, varies also according to the age of the patient, but for a child is proportionately larger than for an adult. We may say that the average dosage recommended is, for an infant, 2,000 to 10,000 units, varying according to the principles mentioned above, and for an adult, 5,000 to 50,000 units. The practice of some clinicians is to give even larger amounts. Thus in cases with extensive false membrane Bie recommended the injection of the serum by both intravenous and intramuscular routes, for a child under ten years of age an initial dose of 80,000 units being given (a quarter to a third of which was injected intravenously), followed at intervals of twelve to twenty-four hours by two further intramuscular doses, the total amounting to 160,000 units or more. It has been claimed that in very severe cases the results of treatment are much improved by such dosage. Without stating the details required for an adequate discussion of the results of antitoxin treatment, it may be said that statistics obtained from all parts of the world afford convincing proof of its advantage, and this is reflected in the practically universal belief of the medical profession in its efficiency. It must be borne in mind that diphtheria antitoxin cannot influence any superadded mixed infection, e.g. with pyogenic organisms. In recent years there has occurred with increased frequency a severe form of diphtheria which responds poorly to antitoxin treatment. According to the observations of Anderson, Happold, McLeod and Thomson in Leeds, the intractable cases are due to infections with the *gravis* form of the bacillus.

ANTIBACTERIAL ANTIBODIES. As with other organisms, the injection into animals of *B. diphtheriae* itself in the living or dead condition, gives rise to antibacterial substances—agglutinins, complement-fixing bodies, etc. Some of these may be present in antitoxic sera, due to the presence of disintegration products of the bacilli in the culture fluid used for preparing the antitoxin;

but there is no evidence that such substances play any part in the therapeutic effects of the antitoxic serum.

SEROLOGICAL TYPES. It is now clear that diphtheria bacilli present considerable heterogeneity as regards their serological reactions and antigenic constitution. On the other hand, all strains yield the same serological type of toxin. The serology of the organisms, as apart from their toxins, has been studied mainly by agglutination tests with antisera, and recently particular attention has been paid to the reactions of strains classified in the first place as *gravis*, *mitis*, and *intermedius*. Agglutination tests are complicated by the difficulty of obtaining uniform suspensions of the organisms and special methods have been adopted to overcome this difficulty. A convenient procedure is to emulsify growths from Löffler's serum medium in 1·5 per cent. sodium chloride and to make the serum dilutions also in 1·5 per cent. saline (Keogh *et al.*; Wright, 1940). Agglutination occurs slowly, and results are read after twenty-four hours at 55° C., the floccules are fine but persist on shaking. The results show that even within the types *gravis*, *mitis*, and *intermedius* there is serological heterogeneity. Orr-Ewing, and Robinson and Peeney have recognised by agglutinin-absorption tests five subtypes of the *gravis* form. One of these is the dominant type in Great Britain. Robinson and Peeney point out that a strain may vary in colony form and starch-

It is now recognised that the diphtheria bacillus is a member of a group of organisms with closely related characters, which are of common occurrence and have a wide distribution as commensals of the skin and mucous membranes of man and the lower animals, and include also specific pathogens of animals. This group corresponds with the genus *Corynebacterium* of the Association of American Bacteriologists, and its characterisation as modified by a Committee of the Medical Research Council may be stated as follows.

'Gram-positive rod-like forms, arranged usually in a palisade, not acid-fast, often with club-shaped swellings at the poles, generally with irregularly stained segments or granules, non-motile, without endospore formation, growing aerobically or under micro-aerophilic conditions, but often capable of anaerobic cultivation, never forming gas in carbohydrate media, in which they may or may not produce acidity, they may or may not liquefy gelatin or serum.'

Members of the genus *Corynebacterium* other than the diphtheria bacillus are usually known in this country as 'diphtheroids'. These have been obtained from the mouth, ear, nose, skin, genital organs, and even from the blood in certain conditions. They are met with in conditions of health, and they have been obtained from many diverse morbid states—from skin diseases, coryza, leprosy, infected wounds, and even from general paralysis bacillus — the differ- ly identical with the diphtheria bacillus both morphologically and culturally, and give the characteristic reaction with Neisser's stain, others, again, differ in essential particulars. The fermentative action on sugars has also been

utilised as a means of distinguishing them ; thus the absence of the power of fermenting glucose or the presence of capacity to ferment saccharose may be accepted as sufficient to exclude the organism from being the diphtheria bacillus. Certain species of diphtheroids produce pseudo-tuberculosis in sheep and mice, ulcerative lymphangitis of horses, and pyogenic lesions in cattle and pigs ; but in general the diphtheroids obtained from the human body are not responsible for any specific pathogenic effects. Further, cultures of these organisms are as a rule quite non-virulent when tested by inoculation of guinea-pigs.

Cases have been recorded, however, in which diphtheroid bacilli have been specifically associated with disease in the human subject. Gilbert and Stewart have described three outbreaks of throat infection, apparently milk-borne, traced to carriers of a diphtheroid bacillus designated '*Corynebacterium ulcerans*'. Schultz *et al.* have reported a case of meningitis due to a diphtheroid bacillus ('*Corynebacterium parvulum*'). Lominski has drawn attention to the occasional occurrence of diphtheroid bacilli in pure culture in the pus of closed suppurative lesions of the finger-pulp (unpublished observations).

Barratt has described a group of 'diphtheria-like' organisms isolated from the human naso-pharynx which, while resembling the diphtheria bacillus, differ from it in certain features and particularly their capacity to liquefy gelatin. They would appear to have some biological relationship to the Preisz-Nocard bacillus, the organism of pseudo-tuberculosis and caseous lymphadenitis of sheep (*vide infra*). Those organisms are virulent to guinea-pigs but their effects are not neutralised, or only partially so, by diphtheria antitoxin. They differ also from the diphtheria bacillus in being pathogenic to rats.

Reference is made later to the classification of the diphtheroid organisms and an account is given of the characters of certain well-defined types.

From what has been said it will be clear that the scientific differentiation of the diphtheria organism may be a matter of some difficulty. With regard to the rules for practical guidance, however, there is general agreement as to the two following. In the first place, in cases of suspected diphtheria the obtaining of a bacillus in a serum culture from the throat, which has all the morphological and staining characters of the diphtheria bacillus, may be accepted as a positive result for all practical purposes. And, further, most will agree that a similar rule should hold in the first instance with regard to bacilli obtained from the throats of immediate contacts. In view, however, of the fact that 'diphtheria-like' bacilli without virulence are present in the throats of some healthy individuals, and may also be present along with virulent bacilli in cases of diphtheria, no one should be regarded as a carrier, dangerous to the community, unless the organism in question is proved to possess virulence. Such a rule rests on the assumption that quite avirulent bacilli do not give rise to infection and the results of the accumulated experience of numerous observers support such a view. In the second place, a 'diphtheria-like' bacillus obtained from another part of the body, with or without a lesion, should not be accepted as etiologically significant, however closely it resembles the diphtheria bacillus, unless it is found on inoculation to produce the characteristic results. On the other hand, an avirulent organism corresponding fully with the diphtheria bacillus in its morphological, cultural, and fermentative character is meanwhile to be regarded as an avirulent diphtheria bacillus.

Bacteriological Diagnosis. (a) *Microscopic examination.* Diagnosis by direct microscopic examination is not advisable, although it may be justified

in cases of urgency when practised by an experienced observer. In some cases the bacilli are present in characteristic form in such numbers as to leave no doubt in the matter. For the purpose films are made from a throat swab, or preferably a piece of membrane if obtainable, and stained with methylene blue and by Neisser's method.

(b) *Cultivation.* The usual routine method is to make cultures from a

detach if possible some of the actual exudate, otherwise it should be thoroughly applied to the tonsils and pharynx in such a way as to soak it in the secretion. Where diphtheritic membrane is present the most satisfactory method is to pick off a fragment with forceps and use it for inoculating the culture medium, but in general, swabs if properly used yield satisfactory results in cases of diphtheria. It is undesirable that much time should elapse between the taking of the swab and the making of cultures, and the drying of the swab owing to delay in transmission to a laboratory may be prejudicial to a diagnostic result. Two methods of cultivation may be adopted and the best results are obtained by combining them: making cultures on Löffler's serum medium in tubes or screw-capped vials, and plate cultivation on a blood tellurite medium, so that separate colonies of the diphtheria bacillus and other viable organisms can be obtained and their characters examined.

(1) The swab is introduced into the tube of Löffler's medium, moistened in the condensation fluid and then smeared over the surface of the serum, being rotated several times in the process. The culture is incubated at 37°C and examined microscopically after eighteen to twenty-four hours. In urgent cases an earlier examination may be carried out, *e.g.* after six to twelve hours. A representative sample of the whole growth is obtained by rubbing a wire loop over the surface, films are made from this and stained by methylene blue and by Neisser's method or a suitable modification of this procedure. Sometimes, when the result is negative, a further period of incubation for twenty-four hours leads to the organisms being found. In clinically suspicious cases a single negative result should not be accepted, but further cultures should be made.

Löffler's serum presents the double advantage over agar that in a mixed culture, such as practically always results from throat swabs, the diphtheria bacilli, owing to their ready growth on this medium, are less likely to be overgrown by other organisms, and in cultures incubated for less than twenty-four hours diphtheria bacilli are more likely to be found. Common throat diphtheroid, Hofmann's type, when stained with methylene blue or by

Neisser's stain is undoubtedly an important auxiliary in the recognition of the diphtheria bacillus, but the results of its use are to be interpreted with caution. Darkly staining granules are not peculiar to the diphtheria bacillus. Some cocci, often giving a metachromatic reaction with methylene blue, may be stained deeply, and other bacilli may contain such granules. A not uncommon organism with such a character occurring in the throat is a streptobacillus with square ends, it has no resemblance to the diphtheria bacillus in a methylene blue preparation, but when stained by Neisser's method may give an appearance very like that organism. On the other hand, a culture of Hofmann's pseudo diphtheria bacillus reacts negatively with Neisser's stain at most a

a Neisser-stained preparation. It is stained with Neisser's staining and in suit for washing the preparations.

The presence of organisms with the characteristic microscopic appearances and staining reactions in a case presenting the clinical features of diphtheria, may be taken as a positive result.

The bacilli does not necessarily exclude diphtheria and that their presence does not in all cases connote 'clinical diphtheria'. In the case of an organism obtained from a suspected carrier or from lesions in parts other than the fauces or larynx, the organism ought to be isolated and further tests applied.

(2) Since cultivation on Löffler's serum and identification by microscopic characters does not provide uniformly satisfactory results, it is now customary to make at the same time cultures on a tellurite medium which, as is generally agreed, may lead to the demonstration of the diphtheria bacillus when negative results are obtained by the former method; and this is particularly true for the *gravis* type. The organisms may be identified by their colony characters, and the *gravis*, *mitis*, and *intermedius* types differentiated. Also, the tellurite medium much more readily yields isolated colonies of the diphtheria bacillus from which pure cultures can be obtained for further tests, e.g. sugar reactions and the virulence test. The two methods used concurrently serve as a check on one another and increase the reliability of diagnostic work. It must be remembered that the tellurite medium is not usually demonstrable.

In case Neisser's staining method is used, tellurite inhibits many other micro-organisms, diphtheroid bacilli are able to grow and must be carefully distinguished from the diphtheria bacillus. McLeod's original blood tellurite medium, as well as others, contains heated blood, and it has been found that certain strains both of *mitis* and *gravis* types are inhibited under these conditions, although they will grow in the presence of unheated blood. Accordingly, unheated blood is preferable for such media, e.g. Hoyle's. For the best differentiation of colony types according to Johnstone and Zinnemann the medium should contain unheated blood along with the low-temperature meat extract of McLeod. For the simple purpose of isolation, Horgan and Marshall's medium is valuable.

The following is a description of results obtained with Hoyle's medium, which yields visible growths even in twelve hours, though eighteen or twenty-four hours' incubation is usually necessary before the colony characters become distinctive; in some cases longer incubation may be required. The appearances noted are those presented after twenty-four to forty-eight hours' incubation.

It does not show distinctly the image of the lamp, the appearance may be of the daisy-head type or more frequently the periphery is irregular, the centre raised and radial striations are observed but sometimes convex circular colonies are seen, the colony is easily broken up by the inoculum, the colour, consistence, and size to *gravis*, and also but convex and having a perfectly circular outline. *Intermedius* type colonies are usually more glistening than those of the diphtheria bacillus, and exceeding 2 mm in diameter, the colonies are usually more glistening than those of the diphtheria bacillus, and under the plate culture microscope and by reflected light, show a smooth surface, the image of the illuminant being often sharply seen on the colony; colour ranges from black

to greyish-white, but the slate-grey of the *gravis* is rarely seen, in consistence, sometimes tough or mucoid. The above descriptions must be regarded only as a guide, and it has to be emphasised that there may be wide variation in the physical characters of colonies, depending on the strain of organism and differences in medium, e.g. its moisture, time during which it has been kept before use, details in the method of preparation, etc.

When the suspected diphtheria bacillus has been isolated its fermentative properties should be determined, glucose and saccharose being used, also starch for identification of the *gravis* type. If the results correspond with

case of absence of fermentation it is important to make sure that growth has occurred in the medium.)

(c) *Virulence test.* For the *subcutaneous method*, the bacillus in question should be grown on a serum slope, the whole of the growth is scraped off and suspended in broth and then injected subcutaneously into a guinea-pig of 250 to 300 grms weight. If the organism is *B. diphtheriæ* the animal will die with the characteristic appearances (p. 224). It is advisable, by way of control, to make at the same time a similar injection into a guinea-pig which has been protected by an intraperitoneal injection, on the previous day, of 500 units of diphtheria antitoxin. The protected animal should show no signs of illness. The *intracutaneous method* is now generally used, as a number of tests can be made on the same animal. The procedure is as follows. Two normal white guinea-pigs of 400 grms weight are selected, one of them is passively immunised on the day before the test by an intraperitoneal injection of 1,000 units of diphtheria antitoxin. They are depilated on the abdomen, then an intracutaneous injection is made into each animal of 0.2 c.c. of a suspension of a pure eighteen to twenty-four hours' culture on Löffler's serum, which has been diluted till its opacity corresponds to about 500 million organisms per c.c. In this way a series of cultures may be tested on one pair of animals, but the injections should be at least 1 inch apart. About six hours after the injections the unprotected animal should receive a 'following dose' of 125 units of antitoxin intraperitoneally in order to prevent its death from toxæmia due to virulent cultures, this dose of antitoxin does not interfere with the development of local reactions. The results are best read after forty-eight to seventy-two hours. A virulent culture causes at the site of injection a small inflammatory patch about 15 mm. in

difficult to obtain a pure culture, what is called the 'immediate' or 'crude' virulence test may be applied. In this case the whole of an impure serum culture containing the organism may be injected subcutaneously, or a portion may be used by the intracutaneous method. As death may be produced by other organisms if present, care must be taken to ascertain that the characteristic effects of *B. diphtheriæ* are produced, and it is essential to use another animal treated with antitoxin as a control.

THE DIPHTHEROID BACILLI

The general application of this designation has already been explained and the differentiation of the diphtheria bacillus from such diphtheroid organisms has been dealt with. Among these organisms certain species

stand out with well-defined and fairly uniform characters, some are specific agents of certain animal diseases and both their biological and pathogenic properties can be clearly determined. But apart from these, many other types of diphtheroid bacilli are met with and attempts have been made to classify them into sub-groups. Results of this work, so far, have not been altogether satisfactory. Morphology shows great variation; metachromatic granules may, or may not, be characteristic; colony characters vary; occasional strains show chromogenesis—yellow, orange, brown, pink; fermentative reactions also show differences. It cannot be said that any classification based on these criteria has proved of practical significance and it is unnecessary to detail here the classifications that have been put forward by different workers (The earlier work on this subject is reviewed in *A System of Bacteriology*, Medical Research Council, 1930, Vol 5, p 136.) Recently, Bray has studied the question further and has utilised an additional criterion, the phosphatase reaction where the organism is grown for twenty-four hours on a phenolphthalein phosphate medium. Many diphtheroid bacilli hydrolyse

the compound with the production of varying degrees of colour (e.g. orange, red, pink) on exposure to ammonia, whereas the diphtheria bacillus has practically no effect. According to colony characters on blood agar and Hoyle's tellurite medium, fermentation reactions and the phosphatase reaction, five sub-groups have been suggested



FIG. 38. Hofmann's (pseudo-diphtheria) bacillus. Young agar culture. Stained with carbol-thionin $\times 1000$.

that put forward earlier by Barratt. Further investigation is required to establish the practical significance of this classification.

The well-defined species, referred to above, will now be described.

Hofmann's Bacillus (*Corynebacterium hofmanni*). This organism, described by Hofmann in 1888, is probably the same as one observed by Löffler in the previous year, and regarded by him as a species distinct from the diphtheria bacillus. It is shorter than the diphtheria bacillus (about 2μ in length) and often shows a somewhat oval shape. When stained by methylene blue it usually exhibits a single unstained area running across it, though sometimes there may be more than one (Fig. 38). A beaded appearance is rarely seen, and the characteristic reaction with Neisser's stain is not given, though in old cultures a few granules which stain deeply may sometimes be found. Involution forms may be produced. It is strongly Gram-positive. It grows readily on the same media as the diphtheria bacillus, but the colonies are larger, thicker, and more opaque. On tellurite serum media colonies are usually circular, glistening, and smooth with a black or grey centre fading to a thin grey periphery; they are often lighter in colour than those of the diphtheria bacillus. It does not form acid from glucose or other sugars, and is non-pathogenic to the guinea-pig. It is usually a relatively easy matter to distinguish this organism from the diphtheria bacillus.

Hofmann's bacillus is of comparatively common occurrence in the throat in normal as well as diseased conditions, including diphtheria; it has been recorded as specially frequent in under-nourished children of the poorer

classes Cobbet found it 157 times in an examination of 692 persons, of whom 650 were not suffering from diphtheria. There is no evidence that Hofmann's bacillus is pathogenic nor can it be transformed into a virulent *B. diphtheriæ* or vice versa, though this was at one time maintained by some.

Bacillus xerosis (*Corynebacterium xerosis*) This name has been given to an organism first observed by Kuschbert and Neisser in xerosis of the conjunctiva. It occurs, however, in many other affections of the conjunctiva and also in the normal condition; in fact, it is a commensal of the normal conjunctiva. Morphologically it is practically similar to the diphtheria bacillus (Fig. 39) and may show metachromatic granules. Even in cultures it presents only minor differences, however, it grows more slowly on serum, and its colonies are small and scaly, have a tough consistence, and a somewhat irregular margin. It produces acid from glucose and also from saccharose. The last property distinguishes it from the diphtheria bacillus. It is non-virulent to animals. It is doubtful whether it has any pathogenic effect in the human subject, though it increases in numbers in abnormal states of the conjunctiva.

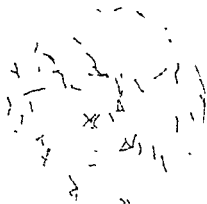


FIG. 39. Xerosis bacillus from a young agar culture. Stained with methylene blue $\times 1000$

Acne Bacillus (*Corynebacterium acnes*). In the pus of acne lesions and also in the comedones a bacillus, generally known as the acne bacillus, may be found in large numbers. The organism was first described by Unna and afterwards cultivated by Sabouraud. It occurs in the form of short rods,

retains the stain in Gram's method. It grows best in the presence of only a small quantity of atmospheric oxygen, being a micro-aerophilic organism, glucose requires to be added to the medium. In shake or stab cultures, growth is at a maximum in a limited zone about half an inch from the surface. It can be cultivated, however, by the usual anaerobic methods. In the substance of glucose agar after three or four days' incubation at 37°C small whitish colonies appear, which when examined under a low magnification have a lenticulate shape. It can also be cultivated aerobically on a serum or blood medium with an acid reaction (pH 6.2 to 6.8). Some observers have obtained surface growth on ordinary agar, especially after the organism has been cultivated for some time under anaerobic conditions. It ferments various carbohydrates, e.g. glucose, maltose, and mannitol. Its relation to the suppuration in acne has been a matter of dispute, some holding that it is the cause of the suppuration whilst others maintain that this is due to pyogenic cocci. The pathogenicity of the organism and its specific relationship to acne is exceedingly doubtful.

Bacillus pseudo-tuberculosis ovis—Preisz-Nocard Bacillus (*Corynebacterium ovis*). This type of organism is responsible for caseous lymphadenitis of sheep and cattle and ulcerative lymphangitis of horses (*pseudo-farcy*). A similar organism has also been found in a pseudo-tuberculous disease of

mice. In morphology little or no distinction can be drawn from the diphtheria bacillus. It grows aerobically at an optimum temperature of 37° C. and best on media containing blood or serum. The colonies on a serum medium tend to show concentric markings, and often develop yellow pigmentation. The growth is rather friable. Gelatin is liquefied and glucose, maltose, and dextrin are fermented. A hæmolysin which lyses the diphtheria bacillus produces a papular lesion which may become pustular in character. Injected subcutaneously it produces death, and its action is not neutralised by diphtheria antitoxin; there is no congestion of the suprarenals and no pleural exudate. The guinea-pig and rat are susceptible to inoculation with cultures; and intravenous injection of the former causes death in about ten days with caseous lesions in lungs, liver and other internal organs. Intraperitoneal inoculation of the male guinea-pig produces infection of the tunica vaginalis, as in experimental infection by the glanders bacillus. Subcutaneous infection of guinea-pigs produces a pyæmic infection.

Bacillus pyogenes (*Corynebacterium*) may also be infected experimentally.

and sometimes almost coccal. Pleomorphism is pronounced. While it may show irregular staining of the protoplasm, metachromatic granules are absent. It is Gram-positive. It grows as an aerobe and facultative anaerobe, but sometimes better under anaerobic conditions. The optimum temperature is 37° C. The best growths are obtained on media containing blood or serum. The colonies on coagulated serum produce small pits of liquefaction, and gelatin is also liquefied. A hæmolysin is produced in culture. Glucose, galactose, lactose, and maltose are fermented. Mannitol is not fermented.

Rabbits

1 pyæmic

are less susceptible. An exotoxin can be separated from cultures and has been shown to be lethal to mice and to produce necrosis of the skin in guinea-pigs. A neutralising antitoxin can be produced by immunisation (Lovell). It is of interest that a fatal case of human infection by this organism has recently been reported in a shepherd (Halbron *et al.*).

Other diphtheroid organisms described in animal diseases are *Corynebacterium renale* and *Corynebacterium equi* (see Merchant). *C. renale* is associated with pyelonephritis of cattle. It differs from the other species described above in the absence of liquefaction of gelatin and coagulated serum. In morphology it is typically diphtheroid with metachromatic granules. On serum medium it produces moist colonies with yellowish pigmentation. Glucose and lævulose are fermented, but not the other carbohydrates which are generally used for fermentation tests. *C. equi* has been described in pneumonia of colts. It is characterised by its abundant viscid growth and red pigmentation. Carbohydrates are not fermented.

CHAPTER VIII

THE TUBERCLE BACILLUS AND THE COMMENSAL AND SAPROPHYTIC ACID-FAST BACILLI (MYCOBACTERIA)

THE cause of tuberculosis was proved by Koch in 1882 to be the organism known as the tubercle bacillus. Probably no other single discovery has had a more important effect on medical science. By the work of Villemin and of Cohnheim and Salomonsen (1870-80) it had been demonstrated that tuberculosis was an infective disease. The latter observers found on inoculation of the anterior chamber of the eye of rabbits with tuberculous material, that after a period of incubation small tuberculous nodules appeared in the iris, afterwards the disease gradually spread, leading to disorganisation of the eye. Later still, the lymphatic glands became involved, and finally the animal died of acute tuberculosis. The question remained as to the nature of the specific infective agent and this question was answered by the work of Koch, which will remain as a classical masterpiece of bacteriological research, both on account of the great difficulties which he successfully overcame and the completeness with which he demonstrated the relations of the organism to the disease. The two chief difficulties were, first, the demonstration of the bacilli in the tissues, and, secondly, the cultivation of the organism outside the body. The tubercle bacillus cannot be demonstrated by a simple watery solution of a basic aniline dye, and it was only after staining for twenty-four hours with a solution of methylene blue with caustic potash added, that he was able to reveal the presence of the organism. All attempts to cultivate it on the ordinary media failed, but he succeeded in obtaining growth on inspissated blood serum, inoculations being made on this medium from the organs of animals artificially rendered tuberculous. The organism was cultivated by the above method from a great variety of sources, and by a large series of inoculation experiments on various animals, performed by different methods, Koch conclusively proved that bacilli from these different sources produced the same tuberculous lesions and were really of the same species.

Tuberculosis is not only the most widely spread of all infective diseases affecting the human subject, and one of the chief causes of death, but there is probably no other disease which affects the domestic animals so widely. The various tuberculous lesions in the human subject need not be described in detail, but some facts regarding the disease in the lower animals may be given, as this subject is of great importance in relation to the infection of the human subject. Amongst the domestic animals the disease is commonest in dairy cattle (bovine tuberculosis), in which animals the lesions are very varied. In most cases the lungs are affected, and contain numerous granulomatous nodules, many being of considerable size; these may be softened in the centre, but are usually of fairly firm consistence and may be calcified. There may be in addition caseous pneumonia, and also small tuberculous granulations. Along with these changes in the lungs, the pleuræ are often affected, and show numerous nodules, some of which may be of large size, firm and pedunculated, the condition being known in Germany as *Perlsucht*, in France as *pommelière*. In other cases, again, the abdominal organs and the peritoneum are principally involved. The udder becomes affected in a certain proportion of cases

of tuberculosis in cows. Tuberculosis is also a comparatively common disease in pigs, in which animals it affects in many cases the abdominal organs, in other cases produces a form of caseous pneumonia, and sometimes is met with as a chronic disease of the lymphatic glands, the so-called 'scrofula' of pigs. In the horse the abdominal organs are usually the primary seat of the disease, the spleen being often enormously enlarged and crowded with nodules; sometimes, however, the primary lesions are pulmonary. In sheep and goats tuberculosis is of rare occurrence, especially in the former animals. It may develop spontaneously in dogs, cats, and in the large carnivora in captivity, also in ferrets. It is met with in monkeys in captivity, and leads to a very rapid and widespread affection, the nodules having a special tendency to soften and break down into a pus-like fluid. Guinea-pigs and rabbits may acquire the infection when kept in the vicinity of animals with open tuberculous lesions. Tuberculosis in fowls (avian tuberculosis) is a common and very infectious disease, nearly all the birds in a poultry-yard being sometimes affected.

The disease in animals thus presents great variations in character, and may differ in many respects from that met with in the human subject. The relations of the different forms of tuberculosis are discussed below, but it may be stated here that two main types of the tubercle bacillus in mammals are now recognised—a *human type* which is the common cause of tuberculosis in the human subject, and a *bovine type* which produces bovine tuberculosis and also a certain proportion of cases of human tuberculosis.

BACILLUS TUBERCULOSIS (*MYCOBACTERIUM TUBERCULOSIS*)

Human Type (*Mycobacterium tuberculosis hominis*). **MICROSCOPIC CHARACTERS.** The bacilli are rod-shaped organisms which usually measure 2.5 to 3.5 μ in length, and 0.3 μ in thickness, i.e. in proportion to their length they are comparatively slender (Figs 40 and 41). Sometimes, however, longer forms, up to 5 μ or more in length, are met with, both in cultures and in the tissues. They are straight or slightly curved, and are either of uniform thickness, or may show slight expansion at their extremities. When stained they appear uniformly coloured, or may present small uncoloured spots along their course, with darkly stained parts between. There is no evidence, as at one time suggested, that such appearances represent spore formation, and it has been shown that 'beaded' bacilli have no higher powers of resistance than those which stain uniformly.

The bacilli in the tissues occur scattered irregularly or in little masses. They are single, or two are attached end to end and often form in such a case an obtuse angle. True chains are not formed, but occasionally short filaments are met with. In cultures the bacilli form masses in which the rods are closely applied to one another and arranged in a more or less parallel manner. They tend to be shorter than those seen in the body. Tubercle bacilli are devoid of motility.

Staining reactions. The tubercle bacillus takes up the ordinary stains very slowly and faintly, and for successful staining one of the most powerful solutions ought to be employed, e.g. crystal violet or basic fuchsin, along with aniline or carbolic acid. Further, such solutions require to be applied for a long time, or the stain being warmed till steam arises, and then the solution being allowed to remain in the hot stain for some minutes. Ehrlich, that after staining has taken place they resist decolorising by solutions which readily remove the colour from the tissues and from

other organisms which may be present. Such decolorising agents are sulphuric or nitric acid in 20 per cent. solution. Preparations can thus be obtained in which the tubercle bacillus alone is coloured by the stain first used, and the tissues can then be coloured by a contrast stain. The bacillus when stained as above for twenty-four hours.

one of the best and
(vide Appendix)

Hot water quickly decolorises stained organisms of the acid-fast group, including the tubercle bacillus. The tubercle bacillus is Gram-positive, but is not readily stained by the usual Gram's method.



FIG. 40. Tubercle bacilli of the human type, from a pure culture on glycerol agar.
Ziehl-Neelsen's stain. $\times 1000$

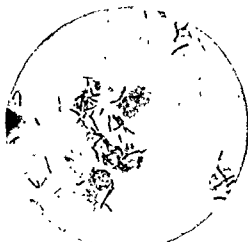


FIG. 41. Tubercle bacilli in phthisical sputum. they are longer than is often the case.
Ziehl-Neelsen's stain and methylene blue
 $\times 1000$

Fluorescence microscopy The tubercle bacillus exhibits fluorescence with certain dyes when examined microscopically by ultra-violet light. It then appears luminous on a dark field. For the purpose an ultra-violet lamp is employed, e.g. a high pressure mercury vapour lamp, with condensing lenses of quartz, and an aluminium mirror in place of the ordinary silvered one. (The microscope condenser may also be of quartz, and the slides of special glass to pass ultra-violet light, but satisfactory results may be obtained without those.) The light is filtered through 'Wood's glass', which excludes visible rays, but, as this allows the transmission of red and infra-red rays, it is also filtered through a solution of 4 per cent. copper sulphate. A yellow contrast filter is placed in the eye-piece. Films are made in the usual way from sputum, etc., stained with auramine solution (0.1 per cent. in 5 per cent. phenol in distilled water) for fifteen

been used as a means of recognising the organism in diagnostic specimens and it has been claimed that positive results may be obtained when Ziehl-Neelsen staining fails, but it is doubtful whether fluorescence microscopy affords any substantial advantage over the ordinary methods.

Aberrant forms In old cultures, for example, very much larger elements may occur. These may be in the form of long filaments, sometimes expanded or clubbed at their extremities, may be irregularly beaded, and may even show the appearance of branching. Their significance has been variously interpreted, for while some look upon them as degenerate or involution forms others regard them as indicating a special phase of the organism.

allying it with the streptothrices. It has also been found that the bacilli in the tissues may produce radiating structures with club-like forms at the periphery closely similar to those of the actinomyces. This was found to be the case when the bacilli were injected under the dura mater and directly into certain solid organs, such as the kidneys in the rabbit. We have observed this appearance in the lungs of rabbits inoculated intravenously with a large dose of the bovine type of tubercle bacillus (Fig. 42). Similar results have been obtained with other acid-fast bacilli and these organisms would appear to form a group closely allied to the streptothrices.

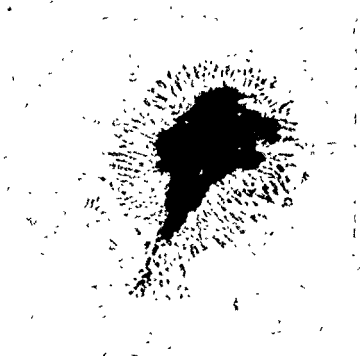


FIG. 42. Large clump of tubercle bacilli (bovine type) in lung of rabbit (experimentally inoculated) showing club formations Ziehl-Neelsen's stain $\times 1,000$.

Granular and non-acid-fast forms Much maintained that the tubercle bacillus may exist in the form of free granules which are demonstrated by longed treatment.

... resemble diptheroids.

It has also been claimed that tuberculous products and cultures of tubercle bacilli may contain the organism in a form which passes through bacterial filters (Vallis) and that in cultures from such filtrates organisms may on occasion be grown which are non-acid-fast but later become acid-fast. On inoculation of the filtrates into guinea-pigs there develops merely an enlargement. Acid-fast bacilli may be present or they may be absent. In fresh animals, in which also the confirmatory results have been obtained.

CHEMICAL COMPOSITION. When the bacilli are extracted with organic solvents such as alcohol, ether, chloroform, or acetone, or mixtures of these, especially with the aid of heat, substances are dissolved out which show the acid-fast property. Tubercle bacilli are relatively rich in these lipoid constituents, but it is extremely difficult to deprive the bacilli of their characteristic staining reaction by such treatment. Aronson found that extraction with a boiling mixture of hydrochloric acid and alcohol removed the acid-fast property, and Bulloch and McLeod by a similar process extracted an acid-fast wax. Long showed that if tubercle bacilli are extracted as completely as possible with alcohol and petroleum ether and are then treated with N/1 HCl a subsequent extraction with the organic solvents removes a further quantity of 'firmly bound' lipoid substances and destroys the acid-fastness and integrity of the bacilli, both of which had resisted the primary extraction. But *B. subtilis* contains practically as much of the firmly bound lipoid although, like other non-acid-fast organisms, its total content in substances soluble in organic solvents is much less than that of the tubercle bacillus. Browning and Gulbransen found that acid-fastness is readily removed at room temperature by treatment with a mixture of chloroform and alcohol containing a minute amount of HCl, e.g. N/100, whereas in the absence of the acid several months' treatment leaves the bacilli still acid-fast. Bacilli which have lost their acid-fast properties by the above methods are still stainable by basic aniline dyes, though they take up the stain feebly.

The facts support Long's view that it is the manner of distribution of the waxy or lipoid substance in the bodies of the bacilli which confers the typical staining properties on tubercle bacilli. This accords also with Koch's observation that the bacilli when disintegrated by trituration cease to be acid-fast.

The investigations of Anderson and his co-workers have further elucidated the nature of the lipoid constituents of the bacillus. By extraction at room temperature of cultures of the human type grown on a synthetic medium, with a mixture of alcohol and ether, followed by chloroform, practically all the materials soluble in neutral solvents are removed. Then by further treatment with a mixture of alcohol and ether containing 1 per cent. hydrochloric acid and final extraction with ether and chloroform, a further portion, the 'firmly bound' lipoids, is obtained, a non-acid-fast residue being left. Thus a series of products is extracted, comprising acetone-soluble fats, phosphatides (acetone-insoluble) and 'waxes' (precipitated from chloroform or ether solution by methyl alcohol). The fats (which are not glycerides) contain various fatty acids, including two higher saturated acids, which are characteristic of the tubercle bacillus, namely tuberculostearic acid and phthioic acid. The phosphatides differ chemically from those of plant and animal origin. The 'waxes' are the most abundant and are chiefly in the chloroform-soluble fraction. They are esters of hydroxy acids with polysaccharides and small amounts of true waxes, etc. On autoclaving with aqueous hydrochloric acid, a part of the waxes is not hydrolysed. This when dissolved in benzene and treated with alcoholic caustic potash yields a polysaccharide and an ether-soluble fraction, which on further saponification liberates a hydroxy acid of very high molecular weight, mycolic acid (the only acid-fast constituent of the tubercle bacillus) and a higher alcohol, phthiocerol - this acid and alcohol are recovered from the other wax fractions also. Phthioic acid and phthiocerol are absent from the firmly bound lipoids. A striking feature is the variable proportion of the several chemical compounds obtained from different strains on the same medium.

In addition, a pigment phthiocol is obtained which is an oxidation-reduction reagent. The fluid of the medium in which the cultures have grown yields several proteins (tuberculo-proteins), which produce the tuberculin reaction (Seibert *et al.*), and in addition polypeptides, which are devoid of tuberculin action, as originally shown by Laidlaw.

and Lan-
after the
form (with
finally dis- singly or *en masse*, the process simulating caseation. (2) Mycolic acid (or the 'wax' containing it) introduced into the tissue powder or in suspension.

f
(3) The only effect of the polysaccharide is that of attracting neutrophilic leucocytes. (4) The effect of the tuberculin is to stimulate the growth of the bacillus.

As to stimulation of monocytes with the tuberculin, it is found that the tuberculin stimulates the growth of endothelioid cells and giant-cells, the latter being both of the foreign-body and the Langhans' types, whereas soluble protein merely produces an accumulation of monocytes. The intensity of the cellular reactions to the proteins is greater in tuberculous animals than in normal. These findings throw some light on the tissue changes observed in tuberculous lesions, and further reference will be made to this subject in relation to the pathogenic action of the tubercle bacillus.

CULTURAL CHARACTERS. The tubercle bacillus is an aerobe and does not grow under strictly anaerobic conditions. Its optimum temperature is about 37° C. and the temperature range for growth is rather restricted, from 30° to 41° C. It cannot be cultivated on ordinary agar or broth media, but primary growths can be obtained on blood or serum media, on pieces of animal tissue or on media containing egg-yolk. After primary culture from the body subcultures of the human type can be obtained on ordinary media to which 5 to 6 per cent. glycerol has been added; the presence of glycerol in other media greatly enhances growth. The medium first used by Koch was inspissated blood serum. If inoculations are made on this medium with the tubercle bacillus, the growth is usually seen as a dull wrinkled film on the surface of the material free from the serum.

minute or raised above the surface of the medium. When compared the appearance of these to that of small dry scales. In such cultures the growths usually reach only a comparatively small size and remain separate, becoming confluent only when many occur close together. In subcultures, however, growth is more luxuriant and may come to form a dull wrinkled film of whitish colour, which may cover the greater part of the surface of the serum and at the bottom of the tube may grow over the surface of the condensation water on to the glass (Fig. 43). The growth is always of a dull appearance, and has a considerable degree of consistence, so that it can be removed from the surface of the medium without the aid of water. In older cultures, when the growth has become confluent on serum, the growth is usually seen as a dull wrinkled film on the surface of the material free from the serum. When colonies are examined under a low power of the microscope, they are seen to

be extending at the periphery in the form of wavy or sinuous streaks which radiate outward, and which have been compared to the flourishes of a pen. The central part shows similar markings closely interwoven. These streaks are composed of masses of the bacilli arranged in a more or less parallel manner.

On *egg medium* and especially on *glycerol egg medium* the human type grows well, producing an abundant dry, irregular, wrinkled or verrucose layer which has usually a yellowish, buff, or pinkish colour. The growth is also characterised by its tough and tenacious consistence. Egg media, particularly when made up with digest broth, are specially suitable for direct cultivation from the tissues.

On *glycerol agar*, which was first introduced by Nocard and Roux as a medium for the culture of the tubercle bacillus, growth takes place in subcultures, but this medium is not suitable for obtaining primary cultures from the tissues, inoculations with tuberculous material usually yielding a negative result. The growth has practically the same characters as on serum. The organism also flourishes well on *glycerol potato*, and this medium has sometimes proved suitable for primary cultures from tuberculous lesions. In *glycerol broth*, especially in a shallow layer in a flask, the bacilli grow readily in the form of little white masses, which fall to the bottom and form a powdery layer. If, however, the growth be started on the surface, it spreads superficially as a dull whitish wrinkled pellicle which may reach the wall of the flask; this mode of growth is specially suitable for the production of tuberculin (*vide infra*). Cultures have a characteristic fruity

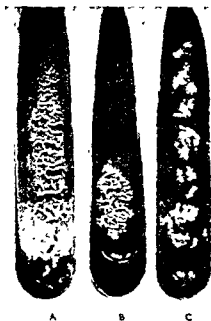


FIG. 43. Cultures of tubercle bacilli on glycerol agar.

(A and B) Mammalian tubercle bacilli of human type. A is an old culture. B is one of a few weeks growth.
(C) Avian tubercle bacillus. The growth is whiter and smoother on the surface than the others.
Natural size.

that their surface is kept moist by the medium, without the fragments being submerged. The growth is probably more rapid and luxuriant than in any other method. A lipoid growth factor for the tubercle bacillus has been extracted from egg yolk and from certain animal tissues by Boissevain and Schultz. Cultures may be readily obtained on fluid synthetic media containing asparagine, ammonium citrate, glycerol, inorganic salts, and a small amount of ferric ammonium citrate (p. 266). The addition of certain surface active agents, especially long chain fatty acid esters of polyhydric alcohols, e.g. polyoxyethylene sorbitan mono-oleate ('Tween 80' of the Atlas Powder Co., Wilmington, U.S.A.), leads to rapid submerged growth of the tubercle bacillus (Dubos). Traces of free oleic acid inhibit multiplication of the organisms, but this action is neutralised by whole serum or undenatured serum albumin (Davis and Dubos). Since the tubercle bacillus grows best

with free access to the oxygen of the air, culture-containers which have been sealed to prevent evaporation should be opened from time to time.

As mentioned growth ceases below 30°C ., but on long-continued cultivation outside the body and in special circumstances growth may take place at a lower temperature. Cultures may remain alive for long periods either at room temperature or at 37°C ., even for several years. Some strains retain their virulence after cultivation for many years, others soon lose virulence.

Bovine Type (*Mycobacterium tuberculosis bovis*). The bovine type is often shorter and thicker and more regular in size than the human type but for practical purposes the two types are morphologically identical (Fig. 44). The growth on various culture media is scantier than that of the human type. From the latter character the Royal Commission applied the terms *dysgonic* to the bovine and *eugonic* to the human type. For distinguishing the growth characters of the two types, egg media are especially suitable. On



FIG. 44. Bovine tubercle bacilli in milk
Ziehl-Neelsen's stain $\times 1,000$

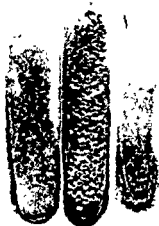


FIG. 45. Cultures of bovine and human tubercle bacilli five weeks old on glycerol egg. The central tube is human, the tubes on each side bovine. The three tubes were inoculated on the same day.
Natural size

such medium the human type, as described above, produces an abundant, dry, and wrinkled or verrucose growth, which has often a yellowish or pinkish tint; while the bovine type forms a thin whitish layer, smooth or somewhat granular, rather

moist in appearance, and the growth is much more easily broken up. The difference between the two types is accentuated by the addition of glycerol to the medium; this greatly favours the growth of the human type, while it does not favour, or even inhibits, the growth of the bovine type. In fact, on glycerol egg medium primary cultures of the latter often fail. On inspissated serum bovine cultures are never pigmented as the human type often is. These differences are most marked in the early cultures; in later subcultures they tend to diminish. The vitality of the bovine type is less on artificial media, cultures having sometimes a tendency to die out. Other differences between the human and bovine types are dealt with later.

Dissociation As first shown by Petroff and his co-workers strains of tubercle bacilli may be dissociated into variants which differ in colony characteristics. These have been designated 'smooth' (S), 'rough' (R), and 'intermediate', according to the appearances of colonies, but it is doubtful whether such variations are strictly analogous to those similarly designated in other bacterial groups. Generally, the colonies of the S type are round

with an almost entire edge, either relatively flat or convex and dome-shaped, smooth and glistening, soft or slimy in consistence and easily emulsified (Fig. 46). The R type colonies are irregular, raised or heaped up and sometimes crater-like, rough and dull in appearance, friable and difficult to emulsify, forming an unstable suspension in physiological saline (Fig. 47). Steenken, Oatway and Petroff have dissociated a virulent strain of the human type into stable 'smooth' and 'rough' forms; the former consisted of flat wrinkled or stippled cream-coloured colonies with spreading veil-like peripheries, the latter of discrete, slightly chromogenic, dry, raised, crater-like colonies which had a clear-cut periphery; the 'rough' type was avirulent, while the 'smooth' form was actively tuberculigenic. It seems possible, however, that this 'smooth' type is in reality an intermediate form between the true S and R. Petroff and his co-workers had found previously that an S variant derived from the avirulent culture of Calmette and Guérin (B C G.—*vide p. 268*) may be pathogenic to laboratory animals

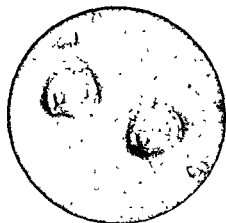


FIG. 46. Colonies of the S type, bovine tubercle bacillus 58 days' growth $\times 10$ (From photograph by Dr M H. Christison)

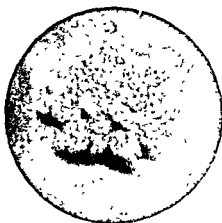


FIG. 47. Colony of the R type, bovine tubercle bacillus 63 days' growth $\times 10$ (From photograph by Dr M H. Christison)

Viability. The tubercle bacillus has considerable powers of resistance to external influences, and can retain its vitality for a long time outside the body in various conditions; in fact, in this respect it may be said to occupy a position intermediate between spores and spore-free bacilli. Dried phthisical sputum has been found to contain virulent bacilli after two months, and similar results are obtained when the bacilli are kept in distilled water for several weeks. So also it resists for a long time the action of putrefaction, which is rapidly fatal to many pathogenic organisms. Sputum has been found to contain living bacilli even after being allowed to putrefy for several weeks and they have been found to be alive in tuberculous organs which have been buried in the ground for a similar period. They are not killed by being exposed to the action of the gastric juice for six hours, they will survive a temperature below freezing point for many months. The bacilli may survive for a time on books or papers read by coughing phthisical persons, but according to Smith there is little likelihood of their remaining viable as long as thirty days, and 'quarantine' of such articles for this time should be an adequate safeguard against spread of infection. It has been found that when completely dried the tubercle bacillus can resist a temperature of 100°C . for an hour, but, on the other hand, exposure in the moist condition to 60°C for the same time is fatal. It may be stated that raising the temperature

to 100° C. kills the bacilli in fluids and in tissues, but in the case of large masses or volumes care must be taken that this temperature is reached throughout. When suspended in watery fluid they are killed in less than a minute by exposure to 5 per cent. carbolic acid; and both Koch and Straus found that they are rapidly killed by being exposed to the action of direct sunlight. But it is much more difficult to sterilise sputum containing the tubercle bacillus by means of antiseptics, as the physical and chemical characters of the sputum prevent effective penetration of the chemicals used. According to Maddock, the bacilli may survive for six months on soil and manure, while on growing grass they may be found alive and virulent for forty-nine days during ordinary summer weather. These findings are of significance in relation to the infection of pastures by tuberculous cattle.

Pathogenic Effects on the Tissues. The *local lesion* produced by the tubercle bacillus is the well-known tubercle nodule, the structure of which varies in different situations and according to the intensity of the action of the bacilli. After the bacilli gain entrance to a connective tissue such as that of the iris, their first action appears to be on the endothelial and connective-tissue cells, which become somewhat swollen and undergo mitotic division,

cells is thus formed in the neighbourhood of the bacilli, and about the same time numbers of leucocytes—chiefly lymphocytes—begin to appear at the periphery and gradually become more numerous. Soon, however, the toxic action of the bacilli comes into prominence. The endothelial cells become swollen and somewhat hyaline, their outlines become indistinct, while their nuclei stain faintly, and ultimately lose the power of staining. The cells in the centre, thus altered, gradually become fused into a homogeneous substance, and this afterwards becomes somewhat granular in appearance. If the central necrosis does not take place quickly, then giant-cell formation may occur in the centre of the follicle, this constituting one of the characteristic features of the tuberculous lesion; or after the occurrence of caseation giant-cells may be formed in the cellular tissue around. The centre of a giant-cell often shows signs of degeneration, such as hyaline change and vacuolation, or it may be more granular than the rest of the cell. The exact mode of formation of a tubercle nodule varies, however, in different tissues.

There can be no doubt that the cell necrosis and subsequent caseation depend upon the products of the bacilli, and are not due to the fact that the tubercle nodule is non-

Reference has been made above to the tissue reactions produced by different chemical fractions of the tubercle bacillus

The *general symptoms of tuberculosis*—pyrexia, wasting, etc.—are to be ascribed to the absorption and distribution throughout the system of the toxic products of the bacilli; in the case of phthisical cavities and like conditions where other bacteria are present, the toxins of the latter also play an important part. The occurrence of amyloid change in the organs is believed by some to be chiefly due to the products of other, especially pyogenic, organisms, secondarily present in the tuberculous lesions.

Presence and Distribution of the Bacilli in Lesions, etc. A few facts may be stated regarding the presence of bacilli, and the numbers in which they

are likely to be found in tuberculous lesions. They are usually very scanty in chronic lesions, whether these are tubercle nodules with much connective-tissue formation or old caseous collections. In caseous material one can sometimes see a few bacilli faintly stained, along with very minute unequally stained granular points; but not infrequently none can be detected. The important fact, however, has been established, that tuberculous material in which no bacilli can be found microscopically may be proved, on experimental inoculation into animals, to be still virulent. In subacute lesions, with well-formed tubercle nodules and little caseation, the bacilli are generally scanty. They are most numerous in acute lesions, especially where caseation is rapidly spreading, for example, in such conditions as caseous pneumonia (Fig 48), acute tuberculosis of the spleen in children, which is often attended with a good deal of rapid caseous change, etc ;



FIG 48 Tubercle bacilli in section of human lung in acute phthisis. The bacilli are seen lying singly and also in large masses. The pale background is formed by caseous material. Stained with Ziehl-Neelsen's stain and methylene blue
x 600

the organisms often form large masses which are easily seen under a low power of the microscope, being specially abundant at the margins of the caseous areas. In acute miliary tuberculosis a few bacilli can generally be found in the centre of the follicles; but here they are often much more scanty than one would expect. The tubercle bacillus is one which not only has comparatively slow growth, but retains its form and staining power for a much longer period than most other bacteria. Its position in the tissue is usually within the cells of the lower animals.

In cattle, for example, the bacilli are commonly found within giant-cells, in which they are often arranged in a somewhat radiate manner at the periphery. It is also common to find bacilli in considerable numbers scattered irregularly throughout the cellular connective tissue of the lesions, even when there is little or no caseation present (Fig 49).

In tuberculosis in the horse the numbers of bacilli may be very great, even in lesions which are not specially acute, and considerable variation

both in their number and in their site is met with in tuberculosis of other animals.

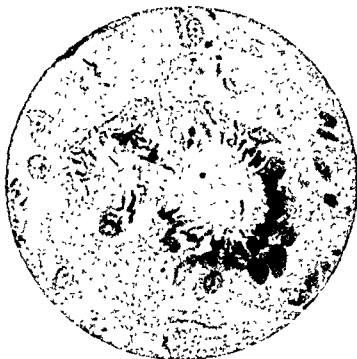


FIG. 49. Tubercle bacilli in giant-cells, showing the radiate arrangement at the periphery of the cells. Section of tuberculous udder of cow. Stained with Ziehl-Neelsen's stain and Bismarck-brown. $\times 1,000$.

In discharges from tuberculous lesions which are breaking down the tubercle bacillus is usually to be found. In the sputum of phthisical patients its presence can be demonstrated almost invariably at some period, and sometimes the numbers are large. Several examinations may, however, require to be made, also animal

inoculation, before any conclusion as to the non-tuberculous nature of a case can be drawn. In tuberculous meningitis the bacilli can often be found in the cerebro-spinal fluid obtained by lumbar puncture, but are frequently scanty. In cases of genito-urinary tuberculosis they are usually present in the urine; but as they are much diluted it is difficult to find them unless a deposit is obtained by means of the centrifuge. This deposit is examined in the same way as the sputum. The bacilli often occur in little clumps, as shown in Fig. 50. In tuberculous ulceration of the intestine their presence in the faeces

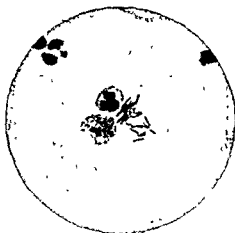


FIG. 50. Tubercle bacilli in urine, showing one of the characteristic clumps in which they often occur. Stained with Ziehl-Neelsen's stain and methylene blue. $\times 1,000$

may be demonstrated, as was first shown by Koch; but in this case their discovery is usually of little importance since the intestinal lesions, as a rule, occur only in advanced stages when diagnosis is no longer a matter of doubt. It should be noted that cultural methods may in some cases reveal

the presence of tubercle bacilli in pathological material in which they cannot be detected by direct microscopic examination.

Claims have been made that tubercle bacilli can be demonstrated by microscopic or cultural methods in the circulating blood in a large proportion of tuberculous cases. Certain of these observations have been possibly fallacious and such findings have not been confirmed (see Wilson, 1933).

Experimental Inoculation. Tuberculosis can be artificially produced in animals in a great many different ways—by injection of the bacilli into the subcutaneous tissue, into the peritoneum, into the anterior chamber of the eye, into the veins; by feeding the animals with the bacilli; and, lastly, by making them inhale the bacilli suspended in the air.

The exact result, of course, varies with the type of tubercle bacillus (human or bovine), and in different animals and according to the method of inoculation, but we may state generally that when introduced into the tissues of a susceptible animal the bacilli produce locally the lesions above described,

the
en-
of
general tuberculosis. Of the animals generally used for the purpose, the guinea-pig is highly susceptible to both types of bacillus.

When a guinea-pig is inoculated subcutaneously with a culture, or with material containing the living bacilli, such as phthisical sputum, a local swelling gradually forms which is usually well marked about the tenth day. This swelling becomes softened and caseous, and may break down, leading to the formation of an irregular ulcerated area with caseous lining. The lymphatic glands in relation to the parts can generally be found to be enlarged and of somewhat firm consistence, about the end of the second or third week. Later, caseous change occurs in them also, and a similar condition may spread to other groups of glands in turn, passing also to those on the other side of the body. During the occurrence of these changes, the animal loses weight, gradually becomes cachectic, and ultimately dies, sometimes within six weeks, sometimes not for two or three months. *Post mortem*, in addition to the local and glandular changes, a general tuberculosis is usually present, the spleen being specially affected. This organ is enlarged and is studded throughout by numerous tubercle nodules, which may be minute and grey, or larger and of a yellowish tint. If death has been long delayed, calcification may have occurred in some of the nodules. Tubercle nodules, though rather less numerous, are also present in the liver and in the lungs, the nodules in the latter organs being usually of smaller size, though sometimes in large numbers. The kidneys only occasionally show nodules. The extent of the general infection varies, sometimes the chronic glandular changes constitute the outstanding feature. Statements as to differences in the pathogenic effects of bacilli from human and bovine sources will be found below.

It has been long recognised that silicosis of the lung predisposes to tuberculosis, and the factors concerned have received considerable study. It was shown by Gye and Kettle that if bacilli were injected along with silica into the subcutaneous tissues of the mouse, the bacilli proliferated readily in the area of coagulation necrosis produced by the silica and seemed to be protected from the tissue defences. Kettle also found that when injected into the circulation of rabbits and mice, they tended to localise in subcutaneous lesions produced by various agents which brought about increased vascularity and tissue necrosis. When silica was used as the provocative agent this localisation was invariable and there was extensive proliferation of the bacilli in the local lesion. Cummins and Weatherall have confirmed Gye and Kettle's findings, but they point out that the effect of silica is local and transitory.

Difference in experimental pathogenicity between the human and bovine types. While in certain animals, e.g. guinea-pig and monkey, there is little difference between the two types, in others a pronounced difference can be established; this is particularly so in cattle and rabbits. Thus the bovine type when injected in suitable quantities into calves produces a local tuberculous lesion, which is usually followed by a generalised and fatal tuberculosis; whereas injection of the human tubercle bacillus usually produces no more than a local lesion, which undergoes retrogression. Corresponding differences emerge in the case of the rabbit, in fact, intravenous injection of suitable quantities (e.g. 0.01 to 0.1 mgm. of young culture suspended in 1 c.c. of saline) in this animal is the readiest method of distinguishing the two types—an acute tuberculosis resulting with the bovine, but not with the human type. In guinea-pigs and monkeys a generalised tuberculosis results from subcutaneous injection of bacilli of the human type, but in this case also the difference in favour of the greater virulence of the bovine type is sometimes made out.

Effects of the Dead Tubercle Bacillus. The interesting fact has been established by independent investigators, that the tubercle bacillus in the dead condition, when introduced into the tissues in sufficient numbers, can produce tubercle-like nodules. Prudden and Hodenpyl, by intravenous injection in rabbits of cultures sterilised by heat, produced in the lungs small nodules in which giant-cells, but no caseation, were occasionally present, and which were characterised by more growth of fibrous tissue than in ordinary tubercle. The subject was very fully investigated, with confirmatory results, by Straus and Gamaleia, who found that if the numbers of bacilli introduced into the circulation were large, there resulted very numerous tubercle nodules with well-formed giant-cells, and occasionally traces of caseation. The bacilli could be well recognised in the nodules by the ordinary staining method. Similar nodules could be produced by intraperitoneal injection. Subcutaneous injection, on the other hand, produced a local abscess, but no secondary tubercles were found in the internal organs. Further, in many of the animals inoculated by the various methods, a condition of marasmus set in and gradually led to a fatal result. These experiments, which have been confirmed by other observers, show that even after the bacilli are dead they preserve their staining reactions in the tissues for a long time, and also that there are apparently contained in the bodies of the dead bacilli certain substances which act locally, producing proliferative and, to a less extent, degenerative changes, and which also markedly affect the general metabolism. Reference has already been made to this question in connection with the chemical constituents of the bacilli. It has been found that animals inoculated with dead tubercle bacilli afterwards give the tuberculin reaction (*vide infra*).

It has recently been shown that the dead bacilli when suspended in liquid paraffin on injection into animals may produce striking effects (Rist). Thus paraffin suspensions of dead organisms of the bovine type injected into the testis of the rabbit, lead to the transport of the bacilli to the lungs, and in this organ produce severe lesions taking the form of a caseous pneumonia, often followed by death (Saenz). At the same time the local lesions produced by paraffin suspensions of the bacilli are more extensive and prone to suppuration. They suggest the destruction of the organisms without isolating them from contact with the tissues. Injection of these paraffin suspensions also brings about intense sensitisation to tuberculin. Such results indicate that the pathogenic action of the tubercle bacillus is intimately related to its chemical or physico-chemical composition.

Bovine Type of Tubercle Bacillus in Relation to Human Disease. This subject first aroused special interest owing to Koch's address at the Tuberculosis Congress in 1901, in which he stated his conclusion that human and bovine tuberculosis were practically distinct, and that if susceptibility of the human subject to the latter really existed, infection was of very rare occurrence—so rare that it was not necessary to take any measures against it. Previously to this, Theobald Smith had pointed out differences between human and bovine tubercle bacilli, the most striking being that the latter possess a much higher virulence for the rabbit and certain other animals, and in particular that the human tubercle bacillus, on inoculation into cattle, produces either no disease or only local lesions without any dissemination. Koch's conclusions were based chiefly on the result of his inoculation of the bovine species with the human type of bacillus, the result being confirmatory of Smith's, and also on the supposition that infection of the human subject through the intestine was of very rare occurrence.

After Koch's communication a large amount of work was done on this subject, and commissions of inquiry were appointed in various countries. We may summarise the chief facts which were established. Practically all observers agreed that there are two chief types of the tubercle bacillus, which differ both in their cultural characters and in their virulence—*human* and *bovine*. The differentiation of these by cultural and virulence tests has already been described. The human type is *eugonic* on glycerol-egg medium, the bovine *dysgonic*, the human type is of low virulence for the rabbit, the bovine produces on inoculation of this animal an acute generalised tuberculosis, and an analogous difference can be demonstrated also in cattle.

Although most of the bacilli which have been cultivated correspond to one of the two types as above described, it is also to be noted that intermediate varieties are occasionally met with, though some of these on analysis have been found to be really due to a mixture of the two types. Griffith has recorded the occasional occurrence of the following aberrant forms—*dysgonic* human, attenuated *dysgonic* human, attenuated *eugonic* human, *eugonic* bovine, attenuated *eugonic* bovine and attenuated *dysgonic* bovine types. The attenuated forms lack the virulence for animals possessed by the

are that this modification does not take place and that the characters of the type are comparatively stable.

With regard to the distribution of the two types, it may be stated that, so far as we know, the bacillus obtained from active bovine tuberculosis is practically always of the bovine type, in fact, this seems to be the prevalent organism in animal tuberculosis (*vide infra*). In human tuberculosis the bacilli in a large majority of the cases are of the human type, but, on the other hand, in a certain proportion the bovine type is present. Pulmonary phthisis is, with few exceptions, caused by bacilli of the human type. Cases have been recorded in which the bovine type has been present, but in general these constitute about 1 per cent. of the cases investigated. In Scotland, however, an incidence of 5 per cent. or over, has been recorded. The Royal Commission found that the bovine type was present in 50 per cent. of cases of primary abdominal tuberculosis in children—that is, in cases where apparently infection had taken place by the alimentary tract, more recent observations have shown that the proportion of cases of glandular tuberculosis in children under ten years of age produced by bovine bacilli varies considerably in different localities. In cases of lupus nearly half of the strains obtained are of the bovine type, and it is an interesting fact that many of

them, both of the human and bovine types, have been found to be markedly attenuated in their virulence for animals. In over two hundred cases of tuberculosis in children, reported by Park (1908-10), the bovine bacillus was present in more than 25 per cent., the percentage being higher in the earlier than in the later years of childhood; and Fraser found that in tuberculosis of bones and joints among children in Edinburgh, this was the type present in more than half. Fraser also found that the proportion of cases in which the bovine type is present is much higher when there is no evidence of infection from other members of the family, than when there is the possibility of such infection. The majority of the tuberculous lesions from which the bovine type has been obtained have been in children. Blacklock in the West of Scotland investigated, *post mortem*, 434 consecutive cases of tuberculosis in children. The pathological evidence pointed to the respiratory system being the portal of entry in 283 (65.2 per cent.); the infecting organisms were isolated in 166, 160 being human strains and 6 (3.6 per cent.) bovine. Primary respiratory tuberculosis was very fatal in children under three years and slightly less so in children between three and thirteen. In 140 the primary site of infection was in the intestinal tract and from these 73 strains were isolated, 13 being human and 60 (82.2 per cent.) bovine. Primary abdominal tuberculosis was less often the cause of death than primary respiratory tuberculosis, but in abdominal infections also, a higher mortality was noted in children under three years. In 9 cases the portal of entry was in the cervical glands (in 7 cases the primary site of infection could not be found, while in 5 there were double primary sites. More isolated from children under two years strains were isolated as follows
18 bovine (64.3 per cent.) ,
and 9 bovine (34.6 per cent.) ; 52 of these strains were isolated during life, and 28 of them (53.8 per cent.) were of the bovine type.

The following collective figures given by Griffith indicate the percentage prevalence of the bovine type of tubercle bacillus in various forms of tuberculosis in this country: cervical gland tuberculosis, 91.3 in England and 65 in Scotland under five years of age; and 50 in England and 52.6 in Scotland at all ages; bone and joint tuberculosis, 19.7 in England and 30.7 in Scotland; meningitis, 24.6 in England and 29.6 in Scotland; lupus, 48.7 in England and 69.2 in Scotland; pulmonary tuberculosis, 1.58 in England and 5.77 in Scotland (the frequency of the bovine type increases from the south to the north of Great Britain, reaching 9.1 per cent. in rural N.E. Scotland); genito-urinary tuberculosis, 17.4 in England and 31 per cent. in Scotland.

In addition to these data regarding the relative prevalence of the bovine type of tubercle bacillus in the different forms of tuberculosis and at different ages, the following facts are of special significance: as estimated, about 30 to 35 per cent. of dairy cows in this country are tuberculous, though only about 0.5 per cent. exhibit udder tuberculosis and actively discharge bacilli in their milk. Recent work indicates, however, that lesions of the udder, detectable by microscopic examination, are much commoner (Stamp). As a result of mixing or bulking, up to 6 or 7 per cent. of samples of raw market milk in the large communities contain the living tubercle bacillus, and this percentage may sometimes be higher. All the evidence points to cows' milk as a serious source of human tuberculosis.

It is of special interest that tuberculous infection of cows is, in most cases, the result of inhalation of the bacilli and involves primarily the lungs, whereas human infection by the bovine type of bacillus is generally due to

drinking cows' milk and involves organs and tissues other than the lungs (see Francis).

Although the bovine bacilli are more virulent to the lower animals than the human bacilli are, this does not hold in the case of the human subject. In fact, the comparative chronicity of the primary abdominal lesions in children, in the first instance, would point rather to a lesser order of virulence towards the human subject. We may also add that there are cases, notably those of Ravenel, in which accidental inoculation of the skin in the human subject with bovine tubercle has resulted in the production of tuberculosis.

It was held at one time that in infancy the susceptibility to infection with the tubercle bacillus is so great that infection once established practically

which makes these views untenable. Of these children 76 died within the first year, one in the second year, and none in the third year; in 72 of them (28.7 per cent. of the total), there was extensive tuberculosis. However, two-thirds of the children survived and in the succeeding three to four years developed well, although many of them showed evidence on X-ray examination of extensive lesions with caseation and calcification of the mesenteric glands. As regards relationship between the site of the primary lesion and the subsequent course of the disease, it is noteworthy that almost all of those who developed extensive primary lung lesions died.

Practical Conclusions regarding the Sources and Modes of Infection. From the facts above stated with regard to the conditions of growth of the tubercle bacillus, its powers of resistance, and the paths by which it can enter the body and produce disease (as shown by experiment), the manner by which tuberculosis is naturally transmitted can be readily understood. The bacilli leave the body in large numbers in the sputum of phthisical patients. As examples of the extent to which this takes place, it may be said that their presence in the air and dust of premises occupied by phthisical patients has been repeatedly demonstrated. Cornet produced tuberculosis in animals by inoculating them with dust collected from hospital wards occupied by phthisical patients. So far as the human subject is concerned, therefore, the great means of disseminating the bacilli in the outer world is dried phthisical sputum, and this source of danger can scarcely be overestimated. The bacilli are also present in the minute droplets expelled from the mouth of the patient when coughing, and these contribute both to the contamination of the air and the dust of premises.

Another great source of infection is the milk of tuberculous cows, especially those affected with tuberculosis of the udder, and this is responsible for a considerable proportion of tuberculosis of lymphatic glands, bones, and joints, etc., in young children as above detailed. The milk from cows thus affected is probably the main source of *tuberculosis mesenterica*, which is so common in young subjects. In these cases there may be tuberculous ulceration of the intestine, or it may be absent. It is especially in children that this mode of infection occurs, since in the adult ulceration of the intestine is rare as a primary infection, though it may result in phthisical patients from bacilli in the sputum which has been swallowed. There is less risk of infection by means of the flesh of tuberculous animals, for, in the first place, tuberculosis involving the muscles of cattle being very rare, there is little chance of the bacilli being present in the flesh unless an affected lymph gland is introduced or the surface has been contaminated with material from tuberculous organs, as in the

process of cutting up the parts ; and, in the second place, even when present they will be destroyed if the meat is thoroughly cooked.

We may state, therefore, that the two great modes of infection are by inhalation and by ingestion of the tubercle bacillus. In the former, the bacilli will in most cases be derived from the human subject ; in the latter, probably from tuberculous cows, though inhaled bacilli may also be swallowed, and contamination of food by tuberculous material from the human subject may occur. Alike when inhaled and when ingested, the bacilli may lodge about the pharynx and thus come to infect the pharyngeal lymphoid tissue, tonsils, etc., tuberculous lesions of these parts being much more frequent than was formerly supposed. Thence the cervical lymphatic glands may become infected, and afterwards other groups of glands, bones, or joints, and internal organs. It is to be noted that there is a predilection for the lungs by whatever route the bacilli enter the body, and accordingly that pulmonary lesions are not always the result of inhalation.

Avian Type of Tubercle Bacillus (*Mycobacterium avium*). In the tuberculous lesions in birds there are found bacilli which correspond in their morphological characters and in their staining reactions to those in mammals, but differences are observed in cultures, and also on experimental inoculation.

Growth takes place at a higher temperature, 43° C., than is the case with mammalian tubercle bacilli. The optimum temperature is 41° to 42° C. On glycerol agar and coagulated serum, growth is more luxuriant and has a moister appearance without the dry scaly character of the human type. On glycerol egg medium the cultural characters may be somewhat similar to those of the human type, but usually the growth is more abundant, smoother, and softer in consistence, and exhibits a creamy yellow or pinkish colour. In glycerol broth the avian type differs from the human in growing mainly in the form of a granular deposit, while sometimes it produces a diffuse turbidity. Most strains grow more rapidly on the various media than the human type. Experimental inoculation brings out even more distinct differences. Tubercle bacilli derived from the human subject or from cattle, for example, when injected into fowls, usually fail to produce tuberculosis, while those of avian origin very readily do so (on the other hand, the parrot is susceptible to inoculation with both mammalian types). Fowls are also very susceptible to the disease when fed with portions of the organs containing avian tubercle bacilli, but they can consume large quantities of phthisical sputum without becoming tuberculous (Straus and Wurtz). Rabbits and mice are the only laboratory mammalian animals susceptible to inoculation with avian tubercle bacilli, though others may succumb to toxic effects when large doses are used. In the case of the rabbit, intravenous injection results in the formation of greyish-white lesions in the spleen, but no true tubercles are formed ; subcutaneous inoculation leads to a peculiar chronic disease in joints, testes, etc., whilst the liver and spleen are free from lesions—a result not obtained with mammalian bacilli. Guinea-pigs which are so highly susceptible to the mammalian tubercle bacilli are resistant to the avian type ; only a local lesion is produced by experimental inoculation.

From analogy we might infer that probably the human subject would be little susceptible to infection from avian tuberculosis ; in fact, only very rare cases have been recorded in the human subject in which the avian bacillus was apparently the causal agent. Nocard stated that mammalian bacilli of the human type, when kept within closed collodion sacs in the peritoneal cavities of fowls over a long period of time, acquired the characters of avian bacilli, but the Royal Commission, as the result of similar experiments, obtained no evidence of such transformation.

Interesting results have been obtained by Winn and Petroff in the dissociation of the avian tubercle bacillus four variants designated 'S', 'FS' (flat smooth), 'R', and 'Ch' (chromogenic) were separated, differing in their physical and chemical properties and as regards the tissue reactions produced by them. As in their studies of dissociation of the human and bovine types, they found that the S forms were the most virulent and the leucocytic response to them was of the acute type, the R form and the chromogenic variant were comparatively avirulent, producing a chronic healing type of lesion.

Human, Bovine, and Avian Types in Tuberculosis of Animals. As has been mentioned, in practically all cases of active tuberculosis of cattle the type of organism is the bovine. This type is also usually found in the horse, sheep, goat, and cat. In the pig the bovine type predominates, but a fair proportion of infections are due to the avian organism and some to the human type, the lesions being of a rather localised form. Both bovine and human types are found in the dog. The human type is found in wild animals in captivity, e.g. chimpanzee, lemur, and monkeys such as *Macacus rhesus*, though in the last-mentioned the bovine type also occurs. The human type has been observed in the parrot, which is susceptible to experimental inoculation with this organism. In recent years special attention has been paid to the occurrence of the avian tubercle bacillus in latent or local retrogressive infections of cattle. Infection of cows has been recorded by Plum in Denmark, and in such cases the uterus was specially affected, with resulting abortion. It seems likely in view of the prevalence of tuberculosis in poultry flocks that dairy herds are exposed to this infection and, though it does not lead as a rule to active disease, the animals become tuberculin-positive, with resulting confusion in the use of the tuberculin test for identifying animals suffering from the usual bovine-type infection (see Harbour).

As has been shown, certain mammalian animals are highly or moderately susceptible, as judged by natural infection or results of experimental inoculation, to both the human and the bovine type, though possessing human type, e.g. cattle, goat, pig, cat, and rabbit. The dog, rat, and mouse are relatively resistant to both types (although in mice the bacilli are present in great numbers in the lesions). The horse is slightly susceptible to the bovine type, but resistant to the human. The parrot and cockatoo are moderately resistant. The various other me, localised,

after several years.

The Vole Type of Tubercle Bacillus. It has been shown by Wells that the field vole (*Microtus agrestis*) is affected in nature by a disease closely resembling tuberculosis and due to an acid-fast bacillus, which may now be classified as a separate type of tubercle bacillus. Great Britain. Morphologically, tubercle bacillus, but is slightly and in the tissues may assume characteristic curved or terminally-crooked forms, in staining reactions it is not differentiated from other types (Brooke). Growth occurs on egg media, but is exceptionally slow, glycerol does not increase the growth. On plain egg media the colonies have been described by Griffith as pearly white and hemispherical, or sometimes frilled, granular, or

umbilicated. In broth, growth occurs in the depth of the medium with, in the case of a trypsin digest broth, a slight surface pellicle. On experimental inoculation of guinea-pigs, rabbits, and calves with moderate doses only local lesions are produced without generalisation of the infection. Rabbits inoculated intravenously with a large dose (1 mgm. of culture) die with appearances like those of acute miliary tuberculosis, but small doses may be non-lethal and produce only scanty tuberculous lesions in the lungs. In guinea-pigs large doses intraperitoneally produce a fatal generalised infection resembling tuberculosis, but doses less than 0.1 mgm. of culture are ineffective. Very large doses subcutaneously bring about only a localised lesion with some involvement of lymph glands. The vole bacillus is non-pathogenic to fowls (Griffith, 1942). By agglutinin-absorption tests with antisera the vole bacillus cannot be differentiated from the human and bovine types, but can be definitely separated from the avian type and the organism of rat leprosy. Infected animals become supersensitive to tuberculin. Wells and Brooke brought forward evidence that inoculation of guinea-pigs with the vole bacillus produces some degree of immunity to virulent human and bovine bacilli, in similar immunisation experiments Griffith and Dalling found that the resistance was only sufficient to delay the progress of the infection; but in calves which received the vole bacillus, they found more definite evidence of immunity. More recently Brooke and Day have reported further experimental data in favour of the immunising properties of the vole bacillus in guinea-pigs. Attention has been drawn by Young and Paterson to severe effects produced in calves, rabbits, and guinea-pigs after inoculation with young cultures of a recently isolated strain of the vole tubercle bacillus. They suggest that no living strain should be used for the purpose of immunisation in man before exhaustive tests have shown the maximum virulence which it can attain.

Persons suffering from tuberculosis show marked cutaneous supersensitiveness to the vole bacillus (living or heat-killed) injected in minute doses intracutaneously, the reaction being analogous to the tuberculin reaction, and guinea-pigs infected with the human tubercle bacillus show a similar reaction. Normal guinea-pigs exhibit no reaction (Cameron and Purdie). It would appear that the specific antigen of the vole bacillus is similar to that of the human and bovine types.

It may be mentioned that the vole is highly susceptible to inoculation with the bovine type of the tubercle bacillus, but relatively insusceptible to the human (Wells, Griffith, 1941).

Piscine Type Tubercle Bacillus (*Mycobacterium piscum*) This organism has been found occasionally in a tubercle-like disease of fish and other cold-blooded animals and is pathogenic to fish, frogs, lizards, and even snails, while possessing no pathogenicity to mammals or birds. Thus, Bataillon, Dubard and Terre cultivated from a tubercle-like disease in a carp, a bacillus which in staining reaction and microscopic characters closely resembled the tubercle bacillus. The lesion with which it was associated was an abundant growth of granulation tissue in which numerous giant-cells were present. This organism, however, formed luxuriant growths at room temperature. Growth did not occur at 37° C., though by gradual acclimatisation a small amount of growth was obtained up to 36° C. This type of acid-fast bacillus in its cultural characters bears a close resemblance to the avian type of tubercle bacillus, growing more rapidly than the mammalian types and producing a more abundant growth which is homogeneous, moist, and creamy, but differs entirely from the avian type in its low optimum temperature and in its non-pathogenicity to birds (Fig. 52, b). Weber and Taute have

cultivated a similar organism from mud, and also from organs of healthy frogs, and it has been suggested by various writers that the bacillus is primarily a saprophyte which sometimes invades the tissues of cold-blooded animals with or without associated lesions. Whether such lesions should be classified as tuberculosis is open to question. Reference will be made later to the saprophytic acid-fast bacilli.

It has been stated by different experimenters that it is possible to modify the human tubercle bacillus by allowing it to sojourn in the tissues of cold-blooded animals, *e.g.* the frog, blind-worm, etc., so that it flourishes at lower temperatures. These results have, however, been called in question, as it has been pointed out that the organisms obtained were other acid-fast bacilli which may be found in the tissues of normal cold-blooded animals.

Allergy in Tuberculosis. The account of the effects of the tubercle bacillus given above shows that it produces toxic substances which both act on the surrounding tissues and also affect the body generally. Nevertheless the organism does not produce separable soluble toxins in fluid media, the toxic substances are bound up with the substance of the bacteria, that is, are endotoxins. Also, in the course of a tuberculous infection, supersensitiveness or allergy is developed toward constituents of the bacillus, *viz.* the specific proteins or protein derivatives of the organisms. This was originally observed by Koch on injecting a second dose of the bacilli into a guinea-pig which had been infected previously. At the site of the second injection an area of acute inflammation and induration developed within two days, the overlying skin became necrotic and later sloughed, with a resulting ulcer which finally healed ('Koch's phenomenon'). The second inoculation was thus ineffective in producing a further tuberculous lesion, while, of course, the original infection was still progressing. Koch also noted that dead bacilli could produce a similar local reaction in a previously infected animal. He concluded that the reaction was due to chemical constituents of the organism and prepared various products from cultures, the so-called *tuberculin*, with a view to obtaining the active constituents in a soluble form. Tuberculous persons and animals exhibit marked allergy to tuberculin, manifested by a *local* reaction at the site of introduction of the tuberculin, by a *general* reaction, indicated by malaise, pyrexia, etc., and also by a *focal* inflammatory reaction around the tuberculous lesions. These reactions accordingly supply a means by which the presence of tuberculous foci in the body can be recognised, this constitutes the diagnostic use of tuberculin. When a sufficiently large dose of tuberculin (0.2 to 0.5 c.c.) is injected subcutaneously into a guinea-pig which has become infected following inoculation with bacilli four or five weeks previously, the animal dies in six to thirty hours and exhibits *post mortem* a severe inflammatory reaction with hæmorrhage at the sites of tuberculous lesions. The condition differs from anaphylactic shock especially in respect of the long interval before death, also passive transference of supersensitive-

being ulcerated and cast off (as in the Koch phenomenon). He strongly advocated tuberculin for therapeutic purposes, and the various forms of tuberculin were produced with a view to getting a suitable preparation. It soon came to be recognised, however, that the administration of tuberculin in the doses then used (doses sufficient to produce a general reaction) might be attended by harm, and the use of tuberculin in this way was abandoned. At a later period treatment by tuberculin was resumed, but was carried out with very much smaller doses, in fact with doses insufficient to produce systemic disturbance, and favourable results have been claimed, though the

therapeutic value of tuberculin has been questioned by many clinical observers. Tuberculin employed in this way might possibly act as a stimulant of the tissue around the tuberculous lesions and thus be beneficial, it might aid also by leading to the production of specific antibodies. But in any case it must be regarded as only an auxiliary agent, and even then only when employed with care and over a considerable period. (For varieties of tuberculin, *vide* p. 266.)

Tuberculin Tests. The original method employed by Koch for eliciting the tuberculin reaction was that of subcutaneous injection, and with it all types of reaction—local, general, and focal—are seen

Subcutaneous method (Koch). A positive reaction may be produced in tuberculous subjects by the injection of 0.001 to 0.0001 c.c. of the 'old' tuberculin (described below). A local inflammatory swelling occurs at the site of injection, and this is accompanied by a rise of temperature, usually of a short character. There is also a focal reaction around any tuberculous lesion, and if the lesion is visible, *e.g.* lupus, this is indicated by an inflammatory redness, which may be followed by ulceration. In the case of a pulmonary lesion there may be signs of irritation—tendency to cough, increased expectoration, increase of râles in the chest, etc. This method is not used now for diagnostic purposes in the human subject.

Cutaneous test of Pirquet. This is carried out as follows: The skin, usually that of the flexor aspect of the forearm, is well cleansed with ether and allowed to dry. Two drops of tuberculin are placed on the prepared surface about 4 inches apart, and then midway between the two drops a small area is scarified with a metal borer constructed for the purpose; in the process only the epidermis should be injured and blood should not be drawn. This serves as a control, any reaction which follows in this being merely a traumatic one. Similar scarification is effected through the drops of tuberculin, so that the scarified spots are exposed to its action. Small portions of cotton wool are placed over the drops to prevent the tuberculin from running off, and the latter is allowed to act for ten minutes. After that time the cotton wool is removed, no dressing is required. The 'old' tuberculin, as originally prepared by Koch, is used. In the case of a positive reaction an inflammatory redness and swelling make their appearance round the sites of tuberculin inoculation, generally within a few hours, and at the end of twenty-four hours there is a distinct inflammatory papule about half an inch in diameter, with a somewhat paler centre like an urticarial wheal; sometimes in the centre there are minute vesicles. The maximum effect usually occurs within forty-eight hours, and after that time the reaction gradually recedes. Such is the typical reaction, but of course slighter, and also more intense reactions are met with. In a negative reaction all three points of scarification show merely a slight traumatic redness which soon passes off.

Intracutaneous method of Mantoux. In this modification 0.1 c.c. of diluted tuberculin is injected into the cutis by means of a fine needle. The method of successive graded injections is specially recommended. For the initial dose a 1:10,000 dilution of tuberculin in 0.5 per cent. phenol-saline is used. Should this fail to cause a positive reaction consisting of an area of erythema or erythematous infiltration not less than 5 mm. in its greatest diameter in forty-eight to ninety-six hours, the test is repeated with a 1:1,000 dilution. If the result be again negative a re-test is carried out with 1:100 tuberculin and, if negative again, with a 1:10 dilution. Re-tests should be made either within a week or after an interval of several months following the previous injection in order to avoid the possibility of sensitisation causing a violent reaction in the intervening period.

The ophthalmic-reaction of Calmette. A dilute solution of 'old' tuberculin, after purification by precipitation with alcohol, is instilled into the eye. A positive result consists in an acute inflammatory reaction of the conjunctiva, which reaches its maximum usually six to ten hours later. Inflammatory reactions of considerable severity may sometimes occur and this method is not to be recommended for routine use.

The general results obtained by these methods appear to correspond closely. A marked positive result is nearly conclusive as to the presence of a tuberculous lesion, though not necessarily of active nature. In cases of latent tuberculosis the reaction is sometimes obtained, sometimes not. Again, in very advanced cases of tuberculosis, especially a short time before death, a negative result may be got, in some of these cases Pirquet met with a colourless papule or a livid spot without exudation, conditions which he described as indicating a 'cachectic reaction'. Also, in measles the skin is temporarily insensitive to tuberculin. The cutaneous and intracutaneous methods are those which have been generally used. The former is the more readily applied, but the latter is the more delicate. The behaviour of the general population in Europe and America has now been extensively investigated by means of the tuberculin reaction. It has been found that while the new-born are insensitive even to large doses, as age advances the number of those who react positively increases steadily until in adult life it becomes very high, reaching 80 per cent or more, according to the particular country and whether an urban or a rural population is examined. These results taken along with the pathological evidence, which tends in the same direction, indicate that a great proportion of the population becomes infected and that, accordingly, the prevalence of tuberculous infection is much higher than purely clinical observations would suggest. The value of the tuberculin reaction as an indication of active infection is therefore greatest in very young children. It is generally agreed that while infections with human or bovine strains react practically equally to tuberculins prepared from either type of tubercle bacilli, they react much less intensely to avian tuberculin and vice versa.

Owing to the very high stability of tuberculin towards heat and the tenacity with which it adheres to apparatus, all syringes and glassware connected with its use should be restricted to this purpose (Farish and O'Brien).

The use of Old Tuberculin in the diagnosis of tuberculosis in cattle. In cattle, tuberculosis may be present without giving rise to obvious symptoms. It is thus important from the point of view of human infection that an early diagnosis should be made. The original method is applied as follows. The animals are kept twenty-four hours in their stalls and the temperature is taken every three hours from four hours before the injection till twenty-four after. The average temperature in cattle is 102.2 F. 30 to 40 centigrams of tuberculin are injected subcutaneously, and if the animal be tuberculous the temperature rises 2 or 3 F. in eight to twelve hours, and continues elevated for ten to twelve hours. Bang, who has done much work on the subject, laid down the principle that the more nearly the temperature approaches 104 F. the more reason for suspicion is there. He gave a record of 250 cases where the value of the method was tested by subsequent post mortem examination. He found that with proper precautions the error was only 3.3 per cent. The method has been largely practised in all parts of the world and is of great value. It may be noted that if a positive reaction is present it may disappear after repeated injections.

The double intra-dermal test introduced by the Tuberculin Committee of the Medical Research Council has been widely accepted as yielding results more trustworthy and easier to interpret than the subcutaneous method. In this procedure a fold of shaved skin near the middle of the animal's neck is taken up between the thumb and forefinger of the left hand and its thickness is measured with calipers. Then 0.1 c.c. undiluted old tuberculin is injected as deeply as possible without penetrating into the subcutaneous tissue. At the end of forty-eight hours the character and thickness of any swelling at the site of injection are noted. A positive result is indicated by a large diffuse swelling with ill defined edges which is hot and tender. If, however, there is only a hard infiltration of the size of a pea or bean without heat or tenderness, the result is inconclusive, and a second injection of the same dose is required. The latter is made at once into the centre of the infiltrated area. The result is read after twenty-four hours. A positive reaction

is shown by an inflammatory swelling with the same characters as those observed in animals which react positively to the first injection. By this method, out of 833 animals which had shown no reaction to the subcutaneous test, 122 reacted positively to the double intradermal test; 91 of the latter came to examination *post mortem*, and all of these were found to be tuberculous.

By the use of the purified protein derivative (*vide infra*) a single intradermal test can now be reliably carried out.

A difficulty has recently come to light in the use of the tuberculin test in cattle due to the fact that these animals may become infected with the avian tubercle bacillus which produces at the most only slight retrogressive lesions. This infection, however, renders the tuberculin test as hitherto carried out, positive. To overcome the difficulty the practice has recently been adopted of testing simultaneously with a bovine and an avian type tuberculin, an animal with an avian type infection reacts more strongly to the avian than the bovine tuberculin.

Varieties of Tuberculin. The following are the most important of a number of preparations which have been used. (1) *Koch's Old Tuberculin*. This consists of a six-weeks-old culture of tubercle bacilli in 5 per cent. glycerol broth, evaporated down to a tenth of its original volume, killed by heat, and filtered.

Standardisation of Tuberculin The potency of preparations of tuberculin is determined by comparing their effect on tuberculous guinea-pigs with that of a standard product.

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of 1 2 000 to 1 4,000. Another method consists in selecting tuberculous guinea-pigs at a stage of the infection when a subcutaneous injection of 0.1 c.c. standard tuberculin kills 50 per cent. of the animals. That concentration of the preparation under test of which 0.1 c.c. subcutaneously produces a similar mortality is taken as equivalent to the standard. The subcutaneous injection of 0.5 c.c. of old tuberculin causes no ill-effects in a normal guinea-pig.

(2) *Tuberculin-O*. Masses of living bacillary growth from surface cultures are dried *in vacuo*, ground in an agate mill, treated with distilled water and centrifuged; the supernatant clear fluid constitutes the tuberculin. As it gave no cloudiness on the addition of glycerol, Koch concluded that it contained the glycerol-soluble products present in the 'old tuberculin', which were looked on as responsible for the necrotic effects produced by the latter (*vide supra*).

(3) *Tuberculin-R*. The deposit in the preparation of tuberculin-O is again ground up in distilled water, centrifuged, and the clear fluid set aside; the process is repeated with the residue until, on centrifuging, none is left. The successive supernatant fluids are mixed and concentrated, and constitute the tuberculin. As this fluid gave a cloudiness with glycerol, Koch considered it contained the glycerol-insoluble constituents of the 'old tuberculin'.

(4) *Koch's New Tuberculin (Bazillénemulsion)*. A bacillary mass is dried and ground in 50 per cent. glycerol in water till a clear fluid results. This tuberculin is thus equivalent to a mixture of tuberculin-O and tuberculin-R.

(5) *Tuberculin from cultures on synthetic media*. By using cultures of tubercle bacilli on synthetic media a tuberculin preparation can be obtained which is free from any non-specific action such as may be produced by proteins derived from the usual culture media (Douglas and Hartley). It has also been shown that the specific protein of the tubercle bacillus, on which the tuberculin reaction depends, can be prepared in purified form and used as a tuberculin for clinical tests (Clark *et al.*; Seibert and Munday). These facts support the specific nature of the active substances in tuberculin. Long and Seibert's modification of Sauton's medium yields an abundant growth of the tubercle bacillus.

Asparagine	5	grms
Ammonium citrate	5	"
Potassium acid phosphate	3	"
Sodium carbonate (anhydrous)	3	"
Sodium chloride	2	"
Magnesium sulphate	1	"
Ferric ammonium citrate	0.05	"
Glycerol	50	"
Water	1,000	"

¹ The *Therapeutic Substances Regulations* prescribe that tuberculin before being supplied commercially must be standardised by an approved method.

✓ *Tuberculin: Purified Protein Derivative (P.P.D.)*. The production of purified tuberculin has been developed by Seibert *et al.*, the resultant product (purified protein derivative) being preserved in a dry state in which form it remains stable for three years at room temperature. Long and Seibert's medium has been used for growing the organism and in this way a preparation analogous to 'old tuberculin' is first obtained. Preliminary purification is

to polysaccharide; the latter is removed by treatment with trichloroacetic acid, the resultant precipitate consisting of the protein derivative; this is separated by centrifuging and dried to a fine powder by a special technique (see Parish; Doig *et al.*).

Immunity. In tuberculosis we meet with certain phenomena unlike those in acute infections against which a solid immunity may be acquired. In the first place, the local reaction around the bacilli plays a very important part, as it represents a struggle between the tissues and the bacilli in which either may be successful. It is now a well-recognised fact that infection and subsequent cure take place in the early years of life in a large proportion of individuals, and that cure may follow even when the lesion has been of considerable extent. The tissue reaction around the bacilli tends to localise them and also their products. The conditions for diffusion of bacterial pro-

assumed that healing of tuberculosis in early life brings with it a certain degree of immunity. Evidence of healed tuberculosis, usually in the lungs, is frequently found in adults, and it is supposed that the appearance of active disease may be due to the lighting up of a dormant lesion. It is also often due, however, to fresh infection, and this is indicated by the fact that in practically all cases of adult tuberculosis the bacilli present are of the human type. In either case, depressed resistance from under-nutrition and like conditions, owing to their overcoming any immunity and leading to susceptibility, are recognised as important factors in the development of active disease. It is possible that the result may vary also according to the virulence of the bacilli, but with regard to this little is known.

The serum of individuals affected with tuberculosis has been investigated for the presence of specific antibodies, and agglutinins, precipitins, opsonins, and complement-fixing immune-bodies have all been demonstrated. The complement-fixation reaction with patients' serum has sometimes been utilised for diagnostic purposes, but is only of limited value, the reaction is usually most pronounced in chronic infections of fairly wide extent, though it may be quite absent when the lesion is encapsulated or quiescent, and also when the disease is advancing rapidly to a fatal termination. Agglutinins obtained by injecting animals with dead bacilli have been employed for the purpose of comparing different strains of bacilli and studying their serological characters. Tulloch and his co-workers found that the strains of the human tubercle bacillus formed one well-defined serological group, there being no evidence of the existence of multiplicity of types. Wilson (1925) came to the same conclusion, and found also that bovine and human strains are indistinguishable by agglutination tests. Thus these two types possess the same specific antigen. Allergic reactions with tuberculin from human and bovine strains also illustrate the antigenic identity of the two types. On the other

hand, the avian type can be differentiated from the mammalian strains by serological and allergic tests and apparently possesses a different antigenic component, while at the same time showing a group reaction due to a common antigenic factor. Ebina has shown by agglutinin-absorption that the acid-fast bacilli from cold-blooded animals and the saprophytic acid-fast bacilli can be distinguished from the human, bovine, and avian types, but draws attention to the group reactions observed by direct agglutination tests among all these organisms. It has been thought that the essential mechanism of immunity may be opsonic in nature and at one time tuberculin was used for therapeutic purposes with the object of increasing the opsonic power of the serum towards the tubercle bacillus, the treatment being controlled by the opsonic index. It should be added that antibodies may result from the injection of large doses of tuberculin in normal animals. But it is not clear whether, and if so to what extent, the presence of antibodies can be taken as an indication of increased resistance to tuberculosis.

It has been generally assumed on the basis of early negative observations with killed-culture vaccines of the tubercle bacillus that to confer any considerable degree of acquired immunity against tuberculosis, it is necessary to inoculate the individual with living, although attenuated, bacilli; and this resistance probably disappears soon after the body ceases to harbour the living organisms. Calmette and his co-workers obtained successful results by immunising animals with a culture of the bovine bacillus which had been attenuated by growth for many years on a medium containing bile, this is generally known as 'B.C.G.' (*Bacille Calmette-Guérin*); and there has been a wide measure of confirmation of their statements (*vide infra*). These findings go to support the view that the healing of a tuberculous lesion in earlier life leads to a certain amount of immunity. They are also in accordance with the fact first brought forward by Koch that a tuberculous animal reacts differently from a normal animal to a fresh infection. In the former the resulting lesions remain localised, while in the latter there may be a spreading infection. It should be noted that according to recent observations on man and experimental animals dead bacilli can stimulate a definite though limited degree of protective immunity, but this is probably not comparable with that produced by the living organism (see Griffith and Glover; Opie *et al*; Wells *et al*). Experimental work of Lurie has indicated that the phagocytes of immunised animals have an enhanced bacteriostatic effect on tubercle bacilli as compared with similar cells of normal individuals; and this is independent of immune-body fluids. Thus 'tissue immunity' may be a factor in acquired resistance to tuberculosis.

Relations of allergy to immunity. Römer showed that tuberculous guinea-pigs were resistant to a small reinfection; and he emphasised that the state of supersensitivity was not necessary for resistance to reinfection. He assumed that

in the same way, though a massive reinfection may overcome the resistance and lead to ulcerative tuberculosis of the lung. Many other observers have also been inclined to correlate resistance with allergy. In this connection it may be noted that dead bacilli produce only a transitory supersensitiveness as compared with the typical allergic condition resulting from infection by living organisms. It has been supposed that the allergic response to a new infection brings about a rapid mobilisation of the same defensive forces which operate in the normal animal. The evidence also indicates that phagocytosis is more active in the allergic individual. On the other hand, certain observers have

claimed that allergy and immunity are not necessarily correlated and that the supersensitive state may, in reality, be an adverse factor (Rich). Thus Sewall and his co-workers have found that in guinea-pigs superinfected after varying times following a primary infection resistance tends to be inversely proportional to cutaneous allergy. Although at present no definite conclusions can be formulated regarding this question and though allergy may not necessarily indicate increased resistance, nevertheless it is clear that allergy represents one phase of the reactive mechanism on which immunity depends. Much may depend also on the degree of allergy, a high degree having an adverse effect by intensifying the local lesion, while a moderate degree may exert a protective action (see Clayson).

Active immunisation of the human subject, etc. In view of the apparent avirulence of his culture B.C.G. (*vide supra*) for the various species of animals susceptible to tuberculosis, Calmette initiated the immunisation of infants with it. Since in the new-born the alimentary mucosa is held to be highly permeable, several doses were given by mouth within the first ten days after birth, the object being to confer protection at the most susceptible period. On experimental grounds, immunity was believed to be well developed a month later. The vaccine has now been extensively applied in France and certain other countries. The original method of administering B.C.G. by mouth has been generally replaced by subcutaneous or intracutaneous injection. Cutaneous administration by 'multiple puncture' has also been specially recommended. The dosage of culture depends on the mode of administration—orally, three doses of 10 mgm. at forty-eight hours' intervals; subcutaneously, one dose of 0.005 to 0.02 mgm.; and intracutaneously, one dose of 0.05 to 0.3 mgm. Objection has been taken to the procedure on the grounds both of its efficacy and its safety. As regards efficacy, favourable statistics published by Calmette have been criticised by Greenwood and others, and it has been concluded that the value of the method is still unproved. On the other hand, striking observations have been recorded by Heimbeck as to its protective value in the case of nurses, originally reacting negatively to tuberculin, who were injected with the vaccine so as to render them positive reactors to the Pirquet test before taking up duty in hospital. Irvine has shown that among over a million vaccinated infants no authenticated death could be attributed to the vaccine, and most of the vaccinated individuals who died and were examined *post mortem* had no pathological signs of tuberculosis. A statistical study of the results of B.C.G. vaccination in young children in Canada has indicated that those treated had a significant advantage as compared with the controls, mortality and morbidity rates being significantly lower (see Hopkins, Baudouin, and Holm).

The immunity to tuberculous infection conferred by B.C.G. on calves and other animals is of special significance and provides so far a better means of assessment of B.C.G. than statistical data in man. Immunisation of calves by B.C.G. has been the subject of a series of observations by Griffith and Buxton and others. In their earlier experiments in which the animals were vaccinated by different routes, alimentary, tracheal, subcutaneous, and intravenous, and were subsequently inoculated intravenously with fully virulent bovine tubercle bacilli, increased resistance was clearly demonstrated after intravenous vaccination, while vaccination by the other routes was of doubtful or variable efficacy. In all the experiments there was great individual variation in the degree of resistance. In subsequent tests in which a double intravenous injection of the vaccine was given, it was found that three months after vaccination the resistance against orally administered virulent organisms was

complete. In later experiments the duration of the increased resistance was estimated: as before, a complete immunity to oral administration of virulent organisms was demonstrated three months after vaccination; at six months the protection was almost complete, but after nine and twelve months a progressive decrease of resistance was noted. Moreover, further vaccination did not completely restore the declining immunity. Buxton and his co-workers have also shown that calves injected intravenously at six-monthly intervals for a period of three years were completely or almost completely resistant to oral administration of virulent bacilli; they found, however, that such resistance was overcome by continued exposure to infection either after the first dose of B.C.G. or after eighteen months had elapsed. It has thus been possible to produce an effective resistance, though of somewhat limited duration, by intravenous vaccination with B.C.G.

As regards safety, the use of a living attenuated culture might seem to involve the risk of virulence being regained. In calves the culture appears to be devoid of pathogenic action (Gerlach), but in small animals such as guinea-pigs tuberculosis may be produced by its injection. Petroff and his co-workers isolated different types of colony from the culture and produced fatal tuberculosis in guinea-pigs; a similar result was obtained by Begbie, who found also that the 'smooth' form was the most virulent (p 251). Dreyer and Vollum claimed that by serial culture of B.C.G. in the depth of a veal broth of pH 6.8 they restored its virulence for the guinea-pig and rabbit. Other workers have failed to separate virulent strains by dissociation from B.C.G. cultures. Lurie has found in experiments with rabbits that the organisms multiply in the tissues but are soon destroyed; some, however, persist in the lymph nodes without producing changes in the tissue. He states that though typical lesions may be produced, these resolve completely. All the evidence points to the fact that in the human subject B.C.G. has no effective virulence. A minor accident attending subcutaneous injections is the development of cold abscesses at the sites.

Spahlinger vaccine. A vaccine of the tubercle bacillus prepared by growing it directly from the body on medium made from the body fluids of the animal species in which the organism has been flourishing under natural conditions has been advocated by Spahlinger. The supposed object is to obtain *in vitro* antigens identical to those generated by the organism in the living body of the infected individual. In addition, no chemical or physical reaction or manipulation is employed which might, as Spahlinger maintains, denature the antigen. The vaccine is prepared by growing the bacilli from the culture are kept for a year or longer. Calves have been carried hardly be regarded as conclusive, though they suggest that a certain degree of immunity is produced by the vaccine.

Anti-Tuberculous Sera. From what has been said regarding immunity reactions in tuberculosis it will be gathered that it is questionable whether the use of passive immunity in the treatment of tuberculosis has a rational basis. Several investigators, however, have introduced for therapeutic purposes the sera of animals treated with the products of tubercle bacilli. The clinical efficacy of these antisera has not been established.

Commensal and Saprophytic Acid-fast Bacilli. A number of species presenting the same staining reaction as the tubercle bacillus have been discovered. Such organisms have a comparatively wide distribution in nature, as they have been obtained from various species of grass, butter and milk, manure, water, and from the surfaces of animal bodies. Microscopically, they correspond more or less closely with the tubercle bacillus, though most of them are shorter and thicker; many of them show filamentous and branching forms under certain conditions of culture. The most important point of

distinction is the fact that their multiplication on artificial media is much more rapid, growth usually being visible within forty-eight hours and often within twenty-four hours at 37° C. Furthermore, in most instances growth occurs at the room temperature, and ordinary agar is a suitable medium. The general character of the cultures in this group is a somewhat irregular layer, often with wrinkled surface, dry or moist in appearance, and varying in tint from white to yellow or reddish-brown.

It is of special interest that, on injection of large doses, they may produce granulation tissue nodules which resemble tubercles, although on the whole there is a greater tendency to softening and suppuration, and usually the lesions are localised to the site of inoculation. These pathogenic effects are more readily produced if the bacilli are injected along with some protective fatty material.

As examples of acid-fast saprophytes we may mention Moeller's grass bacillus (*Mycobacterium phlae*) (isolated from infusions of Timothy grass),

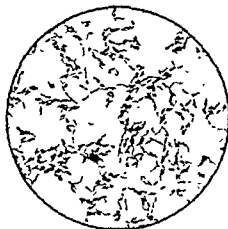


FIG. 51. Moeller's Timothy-grass bacillus.
From a culture on agar.
Ziehl-Neelsen's stain. $\times 1000$.

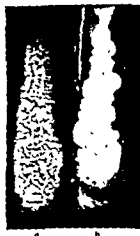


FIG. 52. Cultures of acid-fast bacilli grown at room temperature.

(a) The Petri Rabinowitch butter bacillus.
(b) Bacillus of fish tubercle bac.

Petri's (*M. butyricum*), and Rabinowitch's (*M. berolinsense*) butter bacilli (Figs. 51, 52), the 'Mist (dung) bacillus of Moeller (*M. stercoris*), etc. Kolle and his co-workers have stated that as a result of repeated animal passage certain of the saprophytic acid-fast organisms became enhanced in virulence until they behaved like tubercle bacilli: at the same time their cultural characters also became altered, so that finally they resembled the latter. This has not been confirmed. It may be said that the tubercle bacillus and the acid-fast saprophytic organisms are quite distinct.

Smegma bacillus (*Mycobacterium smegmatis*). This organism is of importance as in form and staining reaction it somewhat resembles the tubercle bacillus and may be mistaken for it. It occurs often in large numbers in smegma and in the region of the external genitals, especially where there is an accumulation of fatty matter from the secretions. Morphologically it is a slender, slightly curved organism like the tubercle bacillus but usually distinctly shorter (Fig. 53). It stains with some difficulty and resists decolorisation with strong mineral acids. Most observers ascribe the latter fact to the fatty matter with which it is surrounded, and find that if the specimen is treated with alcohol the organism is easily decolorised. Zaplewski, however, who cultivated it on various media, stated that in culture it shows

resistance to decolorisation both with alcohol and with acids, and considered,

points of difference in the microscopic appearances of the two organisms are quite sufficient to make the experienced observer suspicious if he should meet with the smegma bacillus in urine, and lead him to apply the decolorising test. Difficulty will only occur when a few scattered bacilli retaining the fuchsin are found, or if they are seen to be enclosed in a matrix of hyaline material. The preparation of stained films after treatment of the urinary

sediment with antiformin is of value in such cases, as organisms which lose their acid-fast properties in the process are not the true tubercle bacillus.

Its cultivation was first effected by Czaplewski. On a serum medium it grew in the form of yellowish-grey, irregularly rounded colonies about 1 mm. in diameter, sometimes becoming confluent to form a comparatively thick layer. He found that it also grew on glycerol agar and in broth. Its cultural characters generally resemble those of the saprophytic acid-fast bacilli described above. It is non-pathogenic to various animals which have been tested, unless very large doses are used.

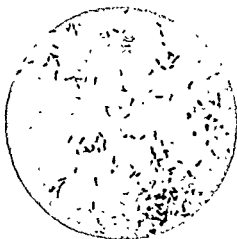


FIG. 53. Smegma bacilli. Film preparation of smegma. Ziehl-Neelsen's stain $\times 1,000$.

Acid-fast bacilli are of common occurrence in the secretions of the external genitals, mammae, etc., in certain of the lower animals, and these organisms vary in character. The term 'smegma bacillus' probably represents a number of allied species.

Bacteriological Diagnosis. The occurrence of saprophytic and commensal acid-fast bacilli must be borne in mind in utilising the reaction of acid-fastness for the identification of the tubercle bacillus. The source of any acid-fast bacilli in question is manifestly of importance, and it may be stated that when these have been obtained from some source outside the body, or where contamination from without has been possible, their recognition as the tubercle bacillus cannot be established by microscopic examination alone. In the case of material coming from the interior of the body, however, the condition must be looked on as different, and although acid-fast bacilli, not the tubercle bacillus or other recognised pathogenic species, have been found on occasions in active lesions in the human subject and in animals (see Laporte), we have not sufficient data for saying that acid-fast bacilli other than the tubercle bacillus and the leprosy bacillus flourish within the tissues of the human body, except in such rare instances as to be practically negligible. Accordingly, up till now, the microscopic examination of sputum, etc., cannot be said to have its validity shaken, and the results of clinical experience show that such examination is of practically unvarying value. Thus tuberculosis is one of the comparatively few diseases in which a diagnosis can usually be definitely made by microscopic examination alone. Nevertheless, the facts established with regard to other acid-fast bacilli must be kept carefully in view, and great care must be exercised when only one or two bacilli

are found, especially if they deviate in their morphological characters from the tubercle bacillus. In such cases animal inoculation may be the only reliable test.

As regards the relative delicacy of the several methods for detecting the presence of tubercle bacilli, it has been shown that in order to demonstrate them by microscopic examination of fairly thick, stained smears of sputum there must be more than 100,000 per c.c. in the specimen, whereas 10 to 100 bacilli (weighing less than one-millionth of a mgm.) of average virulence will

material fail to become infected

(1) *Microscopic examination* In the case of sputum, one of the yellowish fragments which are often present ought to be selected, dried films are then prepared in the usual way and stained by the Ziehl-Neelsen method. When acid-fast bacilli are not found after ten to fifteen minutes' examination of a stained film, instead of prolonging the search, it is advisable to examine further specimens. In cases where the sputum is swallowed as is habitual with young children, washings from the fasting stomach or faeces may be examined, or the examiner's finger, protected by a rubber cap, may be passed to the back of the pharynx and a specimen of sputum so obtained. Alternatively, a throat swab may be used. In the case of urine or other fluids, a deposit should first be obtained by centrifuging. Film preparations are then made from the deposit and treated as before. To avoid risk of contamination with the smegma bacillus, the meatus of the urethra should be cleansed and the urine first passed should be rejected, or the urine may be drawn off with a sterile catheter. As stated above, it is only exceptionally that difficulty will arise to the experienced observer. The best results in the examination of urine are obtained by using the sediment of a twenty-four hours' sample and treating it with antiformin (*vide infra*). Cerebro-spinal fluid may be centrifuged and films made from the sediment, but if the 'spider-web' coagulum (highly characteristic of tuberculous meningitis) forms in the specimen, films should be made from it and these give better results than the centrifuged deposit. The most satisfactory method of examining the coagulum is to withdraw it from the fluid and spread it out on a slide when it can be dried and stained.

The detection of tubercle bacilli by microscopic methods in sputum, pus, faeces, and even tissues, was greatly facilitated by the introduction of the preparation called 'antiformin'. This is a mixture of equal parts of a solution of chlorinated soda (B.P., 1914) and of a 15 per cent solution of caustic soda. It has a remarkable disintegrative and dissolving action on the tissues, etc., so that after it has been allowed to act on sputum, for example, and the mixture is centrifuged, the resulting deposit is scanty and the bacilli, if present, are accordingly greatly concentrated. The time necessary may be judged by the appearance of the mixture, but it will generally be found that the desired liquefaction will be obtained after about an hour if 1 part of sputum be added to 3 or 4 parts of 15 per cent antiformin, the mixture should be kept at 37° C. and shaken from time to time, especially when the sputum

It should be noted that saprophytic acid-fast bacilli may occur in distilled water as used in the laboratory and in tap-water and often in smears from laboratory water-taps. This introduces the possibility of fallacy where water is used for microscopic preparations, e.g. from cultures. Containers for

sputum, etc., should never be closed with corks, as these tend to harbour acid-fast organisms. Further, unused well-cleaned slides should be employed, since bacilli deposited on the glass are difficult to remove.

(2) *Animal inoculation.* The guinea-pig is the most suitable animal for both human and bovine types. If the material to be tested is a fluid, it is injected subcutaneously or into the peritoneum; if solid or semi-solid, it is placed in a small pocket under the skin, or it may be thoroughly broken up in sterile water or other fluid and the emulsion injected. Intramuscular inoculation in the thigh is advantageous, as there is then little tendency for the local lesion to ulcerate. By these methods material in which the tubercle bacillus cannot be found microscopically may sometimes be shown to be tuberculous. Where other organisms are present, preliminary treatment with antiformin is advisable. A negative result in the inoculation test should not be recorded unless the animal has survived six weeks and no lesions are present. Lesions in inoculated animals must always be examined for the presence of the characteristic acid-fast bacilli, since pseudo-tuberculosis produces changes, usually in the liver and spleen, which to the naked eye resemble those of tuberculosis.

The following method of Haswell Wilson for treating tissues is to be recommended. The tissue to be investigated is cut up into small pieces with scissors, and is thoroughly rubbed up in a mortar with a small amount of dry sterile quartz sand. The rubbing up

possible, and the material
mortar are then washed
The sand is allowed to
sediment for a few minutes, as it falls, it carries down with it any coarser particles of tissue which remain. The supernatant fine suspension is then pipetted off, and thoroughly mixed with an equal volume of 15 per cent antiformin. After five minutes, during which it should be stirred continuously, the mixture is centrifuged at high speed for a few minutes, and the supernatant fluid is discarded. The sediment is shaken up with sterile saline solution and again centrifuged. The shaking up of the sediment with sterile saline solution and centrifuging are carried out three times in all, so that no trace of antiformin remains. The sediment resulting from the final centrifuging is used for making cultures (see below), or, after emulsifying with a convenient amount of sterile saline solution, is injected into a suitable animal.

(3) *Cultivation.* The surest method of obtaining pure cultures is to produce tuberculosis in a guinea-pig by inoculation with tuberculous material, and then, killing the animal after four or five weeks, to inoculate tubes of egg medium, under strict aseptic precautions, with portions of a tuberculous organ, e.g. the spleen. The portions of tissue should be fairly large, and should be well rubbed into the broken surface of the medium. Cultures may, however, be obtained from sputum, tissues, etc. after treatment by antiformin as described in (2) above. If, then, inoculations be made from the deposit on egg medium and glycerol egg medium, pure cultures of the tubercle bacillus may be obtained. The method is one which gives good results. The Lowenstein-Jensen medium may be specially recommended for obtaining primary growths of the human type of tubercle bacillus.

Petroff's method (modified) is also recommended as giving satisfactory results. In this, sputum is shaken with three to four times its volume of 4 per cent caustic soda solution, and the mixture is placed for half an hour in the incubator at 37° C., shaking being repeated. At the end of this time it is centrifuged for half an hour at 3,000 r.p.m. The supernatant fluid is then poured off and the sediment carefully neutralised to litmus paper with 8 per cent HCl added drop by drop. The deposit is finally inoculated on egg medium containing 1:10,000 crystal violet.

Corper's method. One c.c. of sputum or other material, in the form of a homogeneous pulp, is mixed in a sterile 15 c.c. centrifuge tube with 1 c.c. of a dilution of sulphuric acid made by adding slowly 17 c.c. concentrated acid, sp.g. 1.84, to distilled water till the final volume is 500 c.c. Mix thoroughly, stopper the tube, and keep at 37° C. for

thirty minutes, shaking several times, then dilute with 10 c.c. sterile 0.85 per cent NaCl solution, mix well, and centrifuge. Make cultures from the sediment. Alternatively, after treatment with acid, the centrifuged deposit is neutralised with caustic soda and cultures then made.

The direct cultivation of the tubercle bacillus from pathological material by the above-described methods has been extensively used for diagnostic purposes and positive results may be obtained when microscopic examination fails to reveal the organism. It must be remembered in using direct cultivation that saprophytic acid-fast bacilli may sometimes be grown from pathological material and that these may be mistaken for the tubercle bacillus. Therefore, if growths do not present quite typical features, the result should be checked by animal inoculation with the cultures.

(4) *Reactive phenomena.* The tuberculin reaction, along with the methods of applying the test, have been described above.

(5) Some use has been made of the *complement-fixation reaction* with patient's serum for diagnostic purposes, though this test has only a limited practical application. The principle of the reaction is similar to that of other complement-fixation phenomena and the system adopted in the Wassermann test may be followed. The antigen is prepared from cultures of the tubercle bacillus (see Coulthard). It should be noted that tubercle antigens may give complement-fixation reactions with Wassermann-positive sera and this possibility must be allowed for by carrying out a parallel Wassermann test.

Identification of type. This depends on the character of growth, as already described, and on the pathogenic action of pure cultures, which are obtained either (1) directly from pathological products, *e.g.* after treatment with antiformin or alkali, or (2) from an animal (guinea-pig) which has been inoculated with such materials. An emulsion of a young culture (two to three weeks) is injected intravenously into rabbits in a dose corresponding to 0.01 to 0.1 mgm. of moist bacilli (or 10 mgm. subcutaneously). Animals which have survived for two months are killed at the end of that time. In the case of *bovine* strains, death usually occurs earlier, *post mortem* an acute generalised infection is found, the lungs especially showing abundant tubercles. *Human* strains commonly fail to kill rabbits within two months, when examined at this time they show at most only scanty isolated lesions in the internal organs (lungs and kidneys). As a rule, dysgonic growth on glycerol egg medium corresponds with high virulence for the rabbit characteristic of *bovine* strains, whereas eugonic cultures show the low virulence of *human* strains. Occasionally, however, there is a lack of correspondence between virulence for the rabbit and cultural characters (p. 257). *Avian* strains may be recognised by their appearance in cultures (moist luxuriant growth) together with their pathogenic action when fed to fowls and lack of virulence for the guinea-pig.

CHAPTER IX

THE LEPROSY BACILLUS: BACILLUS OF JOHNE'S DISEASE

LEPROSY is a disease of great interest, alike in its clinical and pathological aspects; whilst from the bacteriological point of view, also, it presents some striking peculiarities. The disease has a very wide geographical distribution. It occurs in certain parts of Europe—e.g. Norway, Russia, Greece—but is commonest in Asia, occurring in India, Syria, Persia, China, etc. It is prevalent in Africa, in the Pacific Islands, and in certain parts of North and South America. In all these various regions the disease presents the same general features, and the study of its pathological and bacteriological characters, wherever such has been carried on, has yielded similar results.

Pathological Changes in Leprosy. The disease is essentially chronic, a great amount of tissue change occurring without there being necessarily impairment of the general health. In other words, the local effects of the bacilli are well marked, often extreme, whilst the toxic phenomena are proportionately at a minimum unless during exacerbations.

There are two chief forms of leprosy. The one, usually called the nodular or 'tubercular' form—*lepra tuberosa* or *tuberculosa*—is characterised by the growth of granulation tissue in a nodular form, or as a diffuse infiltration in the skin, in mucous membranes, etc., great disfigurement often resulting. In the other form, the anæsthetic—*maculo-anæsthetic* of Hansen and Looft—the outstanding changes are in the nerves, with consequent anæsthesia, paralysis of muscles, and trophic disturbances.

FIG. 54 Section through leprosy
masses of the dark
stained
Ziehl-Neelsen's stain $\times 80$

In the *nodular* form, the disease usually starts with the appearance of erythematous patches attended by fever, and these are followed by the development of small nodular thickenings in the skin, especially of the face, ear-lobes, nose, the backs of hands and feet, and the extensor aspects of arms and legs. These nodules enlarge and produce great distortion of the surface, so that, in the case of the face, an appearance is produced which has been described as 'leonine'. The thickenings occur chiefly in the cutis (Fig 54), to a less extent in the subcutaneous tissue. The epithelium often becomes stretched over them, and an oozing surface then develops, or actual ulceration may occur. The cornea and other parts of the eye, the mucous membrane of the mouth, nose, pharynx, and larynx may be the seat of similar nodular

growths. Nodules in mucous membranes readily ulcerate, and in the nasal secretion large numbers of leprosy bacilli may be demonstrated, this is utilised for diagnostic purposes (*vide infra*). Internal organs, especially the spleen, liver, and testicles, may become secondarily affected. In all situations the change is of the same nature, consisting in an abundant formation of granulation tissue, nodular or diffuse in its arrangement. In this tissue a large proportion of the cells are of rounded or oval shape, like hyaline leucocytes, a number of these may be of comparatively large size, and may show vacuolation of their protoplasm and a vesicular type of nucleus. These are often known as 'lepra cells'. Amongst the cellular elements there is a varying amount of stroma, which in the earlier lesions is scanty and delicate, but in the older lesions may be very dense. Periarteritis is a common change, and very frequently the superficial nerves become involved in the nodules, and undergo atrophy. The tissue in the leprosy lesions is comparatively vascular, at least when young, but, unlike tuberculous lesions, never shows caseation. Some of the lepra cells may contain several nuclei, but cells are not met with resembling in their appearance tubercle giant-cells, nor does a focal arrangement like that in tubercle follicles occur.

In the *maculo-anæsthetic* form the lesion of the nerves is the outstanding feature. These are the seat of diffuse infiltrations, which lead to the destruction of the nerve fibres. In the earlier stages, in which the chief symptoms are pains along the nerves, there occur patches on the skin (*maculæ*), often of considerable size, the margins of which show a somewhat livid congestion. Later, these patches become pale in the central parts, and the periphery becomes pigmented. There then follows a remarkable series of trophic disturbances, in which the skin, muscles, and bones are especially involved. The skin often becomes atrophied, parchment-like, and anæsthetic; frequently pemphigoid bullæ or other skin eruptions occur. Partly owing to injury to which the feet and hands are liable from their anæsthetic condition, and partly owing to trophic disturbances, necrosis and separation of parts are liable to occur. In this way great distortion or mutilation results. The lesions in the nerves are of the same nature as those described above, but the granulation tissue is scantier, and has a greater tendency to undergo cicatricial contraction. This is to be associated with the fact that the bacilli are present in smaller numbers.

BACILLUS OF LEPROSY (*MYCOBACTERIUM LEPRÆ*)

This bacillus was first observed in leprosy tissues by Hansen in 1871, and was the subject of several communications by him in 1874 and later. Further researches, first by Neisser in 1879, and afterwards by observers in various

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both in appearance and in staining reaction. They are straight or slightly curved, and usually occur in groups, though single forms and pairs may be noted (Fig 55). When stained, they may have a uniform appearance, or the protoplasm may be beaded or granular like the tubercle bacillus. The beading is often of a 'coarse' type. They often appear tapered at one or both extremities, occasionally there is slight club-like enlargement. Degenerated and partially broken-down forms are also seen. They take up the basic aniline stains more readily than the tubercle bacillus, but in order to stain them deeply, a powerful stain, such as carbol-fuchsin, is necessary.

When stained, they resist decolorising, though they are often more easily decolorised than the tubercle bacillus; variations, however, exist in this respect, some bacilli losing the stain more readily than others. The bacilli are also readily stained by Gram's method, and in this respect differ from the tubercle bacillus. They are regarded as non-motile and non-sporing organisms. Free granular forms of the leprosy bacillus have been described and regarded as a definite phase in the life history of the organism (*vide* tubercle bacillus, p. 246).

Distribution of Bacilli. They occur in very large numbers in the leprosy lesions, especially in the nodular form—in fact, so numerous are they that the granulation tissue in sections, properly stained as above, presents quite a red colour under a low power of the microscope. The bacilli occur for the most part within the protoplasm of the round cells of the granula-

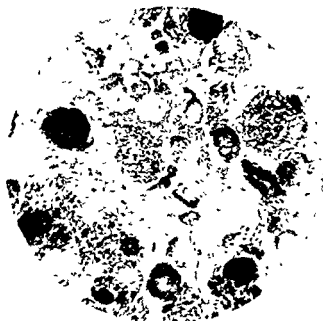


FIG. 55. Leprosy bacilli in smear from nodule in skin, showing the arrangement of the bacilli within granulation tissue cells. Ziehl-Neelsen's stain. $\times 625$.

tion tissue, and are often so numerous that the structure of the cells is quite obscured (Fig. 55). They are arranged in bundles which contain several bacilli lying parallel to one another, though the bundles lie in various directions. The appearance thus presented by the cells filled with bacilli is very characteristic. Bacilli are also found free in the lymphatic spaces, but the greater number are undoubtedly contained within the cells. They are also found in spindle-shaped connective-tissue cells, in endothelial cells, and in the walls of blood vessels. They have been observed in a venous thrombus. They are for the most part confined to the connective tissue, but may be seen in the hair follicles and glands of the skin. Occasionally a few may be found in the surface epithelium, where they probably have been carried by leucocytes, but this position is, on the whole, exceptional. They occur also in large numbers in the lymphatic glands associated with the affected parts. In the internal organs—liver, spleen, etc.—when leprosy lesions are present, the bacilli are also found, though in relatively smaller numbers. In the nerves in the anæsthetic form they are less numerous than in the nodular lesions, and in the sclerosed parts it may be impossible to find any. They are absent from the *macula*, which are essentially trophic lesions

Their spread is chiefly by the lymphatics, though distribution by the blood stream also occurs. They are said to have been found in the blood during the presence of fever and the eruption of fresh nodules, and they have also been observed in the blood vessels *post mortem*, chiefly contained within leucocytes. A few may be detected in some cases in various organs which show no structural change, especially in the capillaries. The brain and spinal cord are almost exempt, but in some cases bacilli have been found even within nerve cells.

Cultivation Experiments. Attempts to cultivate the bacillus have in the majority of cases led to negative results. Further, it seems not unlikely that many of the acid-fast bacilli cultivated from leprosy represent saprophytic non-pathogenic organisms which are widely distributed and may occur on the skin. We have examined a number of the reputed *B. lepræ* strains, and, like others, have found them to resemble closely these non-pathogenic types in their biological characters. Kedrowski isolated an organism which in culture appeared as a non-acid-fast diphtheroid, but which became acid-fast in the tissues of animals. When injected into mice and rats it produced, in a certain proportion of cases, lesions which presented features of human leprosy, the bacilli occurring in large numbers within phagocytic cells. This organism

medium along with amœbæ and symbiotic bacteria, and then, by killing the contaminating organisms by means of heat, obtained a pure growth of a chromogenic acid-fast bacillus. Duval, following up this work, obtained confirmatory results, but in addition to Clegg's bacillus he cultivated a slowly growing non-chromogenic bacillus which he believed to be probably the causal organism. Twort also claimed to have cultivated the leprosy bacillus on glycerol egg medium containing dead tubercle bacilli in the proportion of 1 per cent. Rost and Williams cultivated a pleomorphic streptothrix, which appeared in the form of bacilli or branched filaments, and both of these forms were acid-fast or non-acid-fast. Bayon compared the pathogenic properties of the bacilli cultivated by different workers, and found that only Kedrowski's bacillus and that cultivated by himself, which he regarded as the same

quite clear that certain of the organisms cultivated by various workers and claimed to be leprosy bacilli present essential differences, and some of them correspond closely to saprophytic acid-fast bacillary types.

The successful cultivation of the leprosy bacillus has been claimed by Shiga. Leprosy nodules were triturated with 5 per cent sulphuric acid, and the emulsion was then centrifuged. The deposit was washed with saline solution and again separated in the centrifuge. From it inoculations were made on a potato medium which had been previously boiled in 4 per cent. glycerol broth. After two months, though no visible growth was observed, the bacilli had multiplied and formed masses of well-stained organisms. By subculturing on glycerol agar small visible colonies were obtained.

An acid-fast bacillus has more recently been isolated from leprosy nodules by McKinley and Soule. The cultures were obtained by inoculating a variety

and represented acid-fast bacilli corresponding morphologically to the leprosy bacillus. Monkeys were inoculated intracutaneously with cultures in the supra-orbital region and developed a granulomatous lesion similar to that produced by direct inoculation of material from a case (*vide infra*).^{*} McKinley and Verder later used a medium of minced chick embryo in Tyrode's solution. Growth resulted in a few days and serial subcultivation also proved successful.

Salle has obtained growths from leprosy lesions in chick tissue cultures and obtained subcultures in minced chick embryo medium. The cultures showed both acid-fast and non-acid-fast bacilli, the former being specially abundant when the tissue culture was in active growth. On transferring to ordinary media only the non-acid-fast forms were seen, although on retransferring to tissue medium acid-fast forms reappeared.

Experimental Inoculation. Attempts to transmit leprosy to the lower animals have in general been unsuccessful. Sugai has stated that the Japanese dancing-mouse is comparatively susceptible to inoculation with leprosy material, and Duval has confirmed this observation. The experiments of Kedrowski and Bayon have already been referred to. It is to be noted, however, that in all these cases success was only obtained in a certain proportion, and further that the picture of cells packed with bacilli has also been obtained by the injection of acid-fast saprophytes. In tuberculosis in mice a similar picture is obtained. It would accordingly be a mistake to place much reliance on this point. Experiments have also been performed on monkeys by Nicolle and Blaizot, Reenstierna and others, with inconclusive results. McKinley and Soule have produced granulomatous nodules resembling leprosy lesions by the intracutaneous injection of material from human cases into the supra-orbital region of young monkeys (e.g. *Macacus rhesus*). It would appear that a localised lesion may be produced in monkeys by inoculation with material from human leprosy. This lesion, however, resolves, and there is no spread of the infection. Hamsters seem to be susceptible to inoculation in a proportion of cases.

It would also appear that leprosy is not readily inoculable in the human subject. In a well-known case described by Arning, a criminal in the Sandwich Islands was inoculated with leprosy tissue. Two or three years later, well-marked nodular leprosy appeared, and led to a fatal result. But this experiment is open to the objection that the individual before inoculation had been exposed to infection in a natural way, having been frequently in contact with lepers. Marchoux, however, has recorded the case of a young medical man in Paris whose skin was accidentally pricked by a needle while he was assisting at the operative removal of a skin nodule from a leper. About eight years later he developed leprosy lesions. In other cases, inoculation experiments on healthy subjects and inoculations in other parts of leprosy individuals have given negative results. It has been supposed by some that the failure to obtain cultures and to reproduce the disease experimentally may be partly due to the bacilli in the tissues being dead. According to de Langen also, attempts to superinfect the skin of lepers with material from lesions failed except in some cases where the inocula were derived from patients at the stage of acute exacerbation with fever.

Rat Leprosy. It is interesting to note that a disease occurs under natural conditions in rats which presents many points of close similarity to leprosy. It is very widespread, having been observed in Europe, Asia, America, and Australia; an excellent description was given by G. Dean. In this affection there are lesions in the skin which resemble those in leprosy, and the cells contain very large numbers of an acid-fast bacillus. The disease can be

transmitted to rats by inoculation with tissue material containing the bacilli, but not to animals of other species. The relations of this affection to human leprosy have not yet been established. Bayon claimed to have cultivated the bacillus of rat leprosy, and found that it was practically identical, as regards both cultural characters and pathogenic effects, with the organism obtained from the human disease. It is doubtful, however, whether the organism isolated was the true causal agent.

Allergy and Serum Reactions. It has been found that a considerable proportion of lepers react to tuberculin like tuberculous patients. This result has been variously interpreted, some considering that tuberculosis is also present in such cases, while others maintain that the reaction may be given in the absence of tubercle. If, as is probable, the latter is the case, the result most likely depends on the close relationship of the organisms of the two diseases, it by no means proves their identity. Complement-fixation reactions with patient's serum and culture preparations of various acid-fast bacilli (including strains isolated from the disease, *vide supra*) have been recorded, but their practical significance is doubtful, and they cannot be regarded as possessing any specificity. Another curious fact is that the Wassermann reaction may be given by the serum of leprosy patients (in about 50 per cent, according to some observers). It has been questioned, however, whether the reaction in leprosy is independent of the concurrent presence of syphilis. In a series of cases of leprosy in Malaya, Amies found 28 per cent gave positive Wassermann and Kahn reactions but considered this was not substantially in excess of the syphilis rate in the general population. Further, antisyphilitic treatment caused the reactions to become negative in a large proportion of cases.

Transmission of Disease. The mode by which leprosy is transmitted has been the subject of great controversy. It was at one time considered to be a hereditary disease transmitted from a parent to the offspring. There appears to be no doubt, however, that leprosy subjects may bear children free from leprosy, and that healthy individuals entering a leprosy district may contract the disease. Of the latter occurrence there are well-authenticated instances of persons who contracted leprosy after living and working among lepers. In view of all the facts, there can be little doubt that leprosy is transmitted by

reliable evidence to support the idea

Bacteriological Diagnosis. Film preparations should be made from the discharge of any ulcerated nodule which may be present, or from the scraping of a portion of excised tissue, and should be stained by the Ziehl-Neelsen method substituting 5 per cent sulphuric acid for 20 per cent in decolorising the films. A film should also be stained by Gram's method. The presence of large numbers of bacilli situated within the cells and giving the staining reaction of leprosy bacilli, is conclusive. It is more satisfactory, however, to make microscopic sections through a portion of the excised tissue, when the structure of the nodule and the arrangement of the bacilli can be readily studied. Films of the nasal secretion (*vide supra*) are also examined in the same way, and may yield positive results even in maculo-anæsthetic cases. The points of difference between leprosy and tubercle have already been stated, and in most cases there is really no difficulty in distinguishing the two conditions. A negative result, on inoculating a guinea-pig with the suspected material, will exclude tuberculosis.

BACILLUS OF JOHNE'S DISEASE (*MYCOBACTERIUM PARATUBERCULOSIS*)

An acid-fast bacillus of considerable interest is that of Johne's disease of cattle and sheep, sometimes described as 'chronic pseudo-tuberculous enteritis'. The lesions produced by it take the form of corrugated granulo-matous thickenings of the mucous membrane, especially of the small intestine. The disease has now been observed in various countries, and has been found to be comparatively common in Britain. The bacilli occur in large numbers in the lesions, the cells being often packed with them, and can readily be found in scrapings from the surface. They resemble the other acid-fast bacilli in appearance, but are distinctly shorter than the tubercle bacillus and are usually only 1 to 2 μ in length; they are



FIG. 56. Section of intestine of ox in Johne's disease, showing the cells packed with bacilli. Ziehl-Neelsen's stain $\times 1,000$.

equally acid-fast and alcohol-fast (Fig. 56). The organism was first cultivated by Twort and Ingram on egg medium to which was added $\frac{1}{2}$ to 1 per cent of dried and powdered acid-fast bacilli, the Timothy-grass bacillus being most suitable, growth is slow, the colonies appearing after about four weeks in the primary cultures. The optimum temperature is about 39° C. A filtered glycerol broth culture of the human tubercle bacillus incorporated in egg medium also serves well for the growth of the bacillus of Johne's disease. After the organism has been first grown from the disease, it may often be cultivated on plain egg medium or glycerol broth. The characters of growth are very similar to those of the human tubercle bacillus and on the surface of glycerol broth the growth consists of a dry, wrinkled pellicle. It has been shown that plant extracts may also contain the growth factor which this organism requires. Experimental inoculation of calves (especially by the intravenous route) reproduces the disease after a long incubation period, but small laboratory animals are not susceptible. 'Johnin' prepared from cultures of the organism in the same way as tuberculin has been used as a specific diagnostic agent, but the specificity of the reaction with this product has been questioned.

CHAPTER X

THE ANTHRAX BACILLUS

ANTHRAX is a disease occurring epizootically among the herbivora, especially sheep and cattle, in which animals it has usually the characters of a rapidly fatal septicæmia with extensive multiplication of the bacilli in the blood, and splenic enlargement. The disease is comparatively rare in the human subject and does not occur as a natural infection from man to man, but may be communicated to him directly or indirectly from animals. In the human subject, the bacilli are much more restricted to local lesions than is the case in the ox

Historical researches leave little doubt that from the earliest times anthrax has occurred among cattle. For long its pathology was not understood, and it went by many names. In 1849 Pollender observed that the blood of anthrax animals contained numerous rod-shaped bodies which he conjectured had some causal connection with the disease. In 1863 Davaine announced that they were bacteria. He stated that they appeared in large numbers in the blood a few hours before death and that unless blood used in inoculation experiments on animals contained them death of the latter did not ensue. Though this conclusion was at first disputed, still by the work of Davaine and others the causal

culture outside the body, and, by inoculating animals with them, produced the disease artificially. Koch's observations were, shortly afterwards, confirmed in the main by Pasteur, though controversy arose between them on certain minor points. Moreover, further research showed that the disease could be produced in animals by feeding them with spores, and thus the way in which the disease might spread naturally was explained. Koch's work on anthrax led to the development of the present-day methods of cultivation and isolation of bacteria, while that of Pasteur provided a means of preventing the disease by active immunisation.

BACILLUS ANTHRACIS

MICROSCOPIC CHARACTERS. If a drop of blood is taken from an auricular vein of an ox immediately after death from anthrax, it will be found to contain a great number of large non-motile bacilli in pairs or short chains. On staining with watery methylene blue, the characters of the bacilli can be better made out. They are about 5 to 8 μ long and 1.2 μ broad, though both shorter and longer forms also occur. The ends are sharply cut across, or may be slightly concave so as to resemble somewhat the proximal end of a phalanx. In cultures similar appearances are observed, but chain formation is more marked. The bacillary protoplasm is very finely granular, and very frequently appears surrounded by a capsule, whose external margin, however, is often not so well defined as in the case of the pneumococcus. When several bacilli lie end to end in a chain, the capsule seems common to the whole chain. They stain well with all the basic aniline dyes and are Gram-positive. The capsule can be demonstrated by the usual methods, including relief staining (Fig. 58).

A simple method for the demonstration of capsular material when the organism is present in blood or body fluids was introduced by McFadyean—

and whorled, which produce a star-like form. These spikelets are -- 1
of spirally twisted threads.

In gelatin stab --

when a low -- present, and when the
tube is dire -- infected blood. In about two days there
radiate out into the medium from the wire track numerous very fine
spikelets which enable the cultures to be easily recognised. These spikelets
are longest at the upper part of the track; thus the
'inverted fir tree' appearance results (Fig. 61). Spread
takes place on the surface of the gelatin, and here
liquefaction commences, and gradually extends down the
stab and out into the medium, till the whole of the
gelatin may be liquefied.

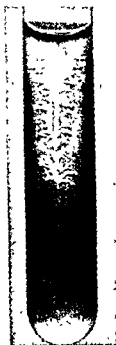


FIG 61. Stab culture of the anthrax bacillus in peptone-gelatin, seven days' growth. It shows the 'spiking', and also, at the surface, commencing liquefaction. Natural size.

The organism grows readily on *potato*, but the cultures show no special characteristics. *Coagulated serum* is slowly liquefied. On *blood agar* the organism is weakly hæmolytic or non-hæmolytic; but results depend on the blood incorporated in the medium and the strain examined. Some strains are lytic to sheep blood, but horse blood is not usually affected.

In *broth* after twenty-four hours' incubation at 37° C., there is usually the appearance of irregular spiral threads suspended in the liquid, which are made up of bundles of parallel chains of bacilli. Later, growth is more abundant and forms a flocculent deposit in the tube.

While the above are the typical cultural appearances met with in virulent cultures, *variants* may occur either spontaneously or as the result of growth under special conditions (*vide infra*).

BIOCHEMICAL REACTIONS. The anthrax bacillus ferments glucose, saccharose, and maltose with acid but no gas formation.

VIABILITY AND CONDITIONS NECESSARY FOR SPORULATION. In the spore-free condition the bacilli have comparatively low powers of resistance. They do not stand long exposure to 60° C., and if kept at ordinary temperature in the dry condition they are usually found to be

dead after a few days. The action of the gastric juice is rapidly fatal to them, and they are accordingly destroyed in the stomachs of healthy animals. They are also soon killed in the process of putrefaction. They can be cooled below the freezing-point without dying.

Spores, as contrasted with the vegetative organisms, possess a high degree of resistance to various adverse influences. Koch found they resisted boiling for five minutes, and dry heat at 140° C. must be applied for several hours to kill them with certainty. Graham-Smith reported that in a dry condition at ordinary room temperature and exposed to diffuse daylight 50 per cent. of spores cease to be capable of germinating in a few months, while a considerable proportion of the remainder is still able to germinate after ten years. Some spores remain viable for as long as twenty-two years. Unlike the bacilli, they can resist the action of the gastric juice for a long period of time. It is to be noted that spores derived from a given culture possess varying resistance among themselves, also the spores formed by different strains of the organism may vary markedly in this respect.

When present on hides spores survive lime tanning, but on the addition of 1 per cent. sodium sulphide to the lime bath, provided the temperature is not below 23° C., they are unlikely to resist ten days' treatment (Robertson). According to Kast, chrome-alum as used in 'chrome-tanning' takes twenty-seven days to kill spores. Egorov recommends 10 per cent freshly prepared chloride of lime for the disinfection of certain appliances used in the tanning process, the same concentration of caustic soda or of commercial hydrochloric acid for other appliances. These have been tested successfully with equipment heavily contaminated artificially with spores from a resistant strain.

It is generally agreed that sporulation never occurs within the body of an animal suffering from anthrax. Koch attributed this to the absence of free oxygen, which he found necessary to the occurrence of spores in cultures outside the body. Another factor is necessary to sporulation, namely, a suitable temperature. The optimum temperature for spore production is 30° C. Koch found that spore-formation did not occur below 18° C. Above 42° C not only does sporulation cease, but Pasteur found that if bacilli were kept at this temperature for a time they did not regain the capacity when again grown at a lower temperature. In order to make them again capable of sporing, it was necessary to adopt special measures, such as passage through the bodies of a series of susceptible animals. Non-sporing derivatives may also be isolated from sporing strains, especially when those opaque portions of growth are selected which develop late on old agar cultures.

is favoured by

VARIATION

variation. In some cases it is a well-defined structure like that of the pneumococcus, but it may lack definition of its external contour, and laboratory strains of the organism may be devoid of capsular material altogether. It has been shown that capacity to produce a well-formed firm capsule *in vivo* is associated with the possession of special virulence, non-capsulate strains frequently showing weakly pathogenic qualities. In this connection it is important to remember that the capsulate forms are insusceptible to phagocytosis, while the non-capsulate are readily taken up by phagocytes. The capsule consists of a polypeptide of *D*-glutamic acid, but is free from polysaccharide. On the other hand, polysaccharide can be demonstrated in the bodies of non-capsulate as well as capsulate anthrax bacilli (Tomesik and Szongott). Variations in capsule formation are also associated with differences in the naked-eye appearance and physical characters of cultures. In those where the capsule is indefinite, the growth is moister and

heat, and different colonies isolated from such strains may present differences in the character of the capsule.

Highly virulent strains present the typical cultural appearances already described, i.e. on agar the colonies are large, 'rough', wreathed at the edge, and made up of bacilli in continuous chain formation. In laboratory cultures variants may be found presenting a smaller and 'smooth' type of colony without the typical wreathed margin, the bacilli being arranged in pairs or bundles; they are less virulent than the rough type. This type of colony is characteristic of cultures which have been attenuated by growth above the optimum temperature, as in Pasteur's method of producing anthrax vaccine (*vide infra*). Repeated subculturing of the rough type on agar containing

calcium salts leads to diminution in sporing forms and finally to a filamentous non-sporing strain which is avirulent. The growth of this non-sporing organism on media decalcified by the addition of oxalate leads to the formation of a mucoid variant.

It is doubtful, however, if the terms 'rough' and 'smooth' apply here in the same sense as to the Gram-negative intestinal pathogens. When the anthrax bacillus is grown on horse serum solidified by heating at 75° C. smooth colonies are found to be capsulate and virulent, while rough colonies show little capsule formation and are practically non-virulent (Takahashi).

It is evident from what has been said that modifications, in both biological and cultural characters can be artificially originated in anthrax bacilli. Spores which are kept in the dry state under ordinary conditions for many months, e.g. by letting an agar culture dry up, usually yield virulent cultures. A good method of preserving the virulence and sporing property of *B. anthracis* is to maintain it in cultures on agar containing 0.1 to 0.2 per cent. neutral sodium oxalate to act as a decalcifying agent (Bordet, *vide supra*).

Toxic Properties. It must be recognised that the manner in which the anthrax bacillus produces its pathogenic effects is still imperfectly understood. At one time it was thought that blockage of capillaries by the bacilli multiplying in the blood might be a lethal factor, but this would not in itself explain the pathogenic action of the organism in all cases. Toxic action is undoubtedly concerned, as is indicated by the inflammatory oedema (*vide infra*) which occurs apart from the actual presence of the bacilli, and by the general toxic effects observed in man and animals suffering from the disease, but it has not, so far, been possible to separate toxins from cultures. Filtered broth cultures are almost non-toxic and the dead bacilli themselves have little toxic effect. The facts would suggest that the bacilli when growing in the tissues form toxins such as are not produced in cultures. Bail pointed out that, in the body, an 'aggressin' is produced by the organism, which enhances its invasiveness. This aggressin was obtained by centrifuging oedema fluid from infected animals and then killing any remaining bacilli. It seems possible that the capsular substance of the anthrax bacillus (cf. pneumococcus) acts as an aggressin and protects the organism from phagocytosis by combining with serum opsonins. As has been pointed out, virulence is associated with capsule formation. It is not, of course, necessary in the animal disease to postulate a toxin of very high potency, in view of the fact that in fatal cases the bacilli are present in exceedingly large numbers in the blood and tissues. It is of interest to note that Aoki and Yamamoto claim to have demonstrated in cultures an endotoxin which is thermolabile and therefore would not be demonstrable when cultures killed by heat in the usual way are injected into animals. At present no definite statement can be made regarding the nature of the toxic action of the organism and the toxic substances it produces in the body or in culture medium.

Anthrax in Animals and Experimental Inoculation Anthrax occurs from time to time epizootically in sheep and cattle, and may also attack goats, horses, pigs, deer, and camels; it is world-wide in its distribution. It occurs throughout Europe, but in some countries it is much more common than in others. In Britain the death-rate is small, and often only one animal in a herd is affected, but in France the annual mortality among sheep was formerly about 10 per cent. of the total number in the country, and among cattle 5 per cent. The incidence, however, has been greatly reduced—a result ascribed to the system of preventive inoculation (*vide infra*). In sheep and cattle the disease is specially virulent, and death often occurs with great rapidity. In less acute cases the animal is apparently out of sorts, and does

not feed, there is often a profuse discharge of mucus from the nostrils, and the mucus may be tinged with cyanide.

When the disease is more prolonged, widespread œdema and extensive enlargement of lymphatic glands are marked features, and in the glands, especially about the neck, actual necrosis with ulceration may occur, constituting the so-called anthrax carbuncles. Such subacute conditions are especially found among horses, which are by nature not so susceptible to the disease as cattle and sheep. In pigs, marked inflammatory œdema of the tissues around the pharynx is a feature. In affected cows the bacilli may be present in the milk. Occasionally even in susceptible animals recovery takes place. It is in the terminal stage of the disease that the organisms usually attain great numbers in the blood.



FIG. 62. Scraping from spleen of guinea-pig dead of anthrax, showing the bacilli mixed with leucocytes, etc. (Same appearance as in the ox.)

Wet fixed film stained with carbol thionin $\times 1,000$

On post-mortem examination of an ox dead of anthrax, the most noticeable feature—which has given the name 'splenic fever' to the disease—is the enlargement of the spleen, which may be two or three times its natural size. It is of dark red colour, and on section the pulp is very soft and friable. A film made from the spleen and stained with watery methylene blue will be found to contain enormous numbers of bacilli mixed with red corpuscles and leucocytes, chiefly lymphocytes and the large mononucleated variety (Fig. 62). The lymphatic system generally is much affected, especially in less acute cases. The glands, especially the mediastinal, mesenteric, and cervical glands, are enlarged and surrounded by œdematous tissue, the lymphatic vessels are

The
mated,
ins are

those met with in septicæmia. The blood throughout the body is usually fluid and of dark colour; bacilli can be found in it on microscopic examination, but they are specially numerous in the capillaries of internal organs.

Great differences exist in susceptibility to anthrax in different species of animals. Thus the sheep (except those of Algeria, which, under experimental conditions, only succumb to very large doses of the bacilli), guinea-pig, and mouse are all very susceptible, the rabbit slightly less so. Cattle are highly susceptible to the natural infection, but much less so to subcutaneous inoculation. Less susceptible than this group are the horse, deer, and goat, in which the disease occurs from time to time in nature. Anthrax in the pig occurs epizootically, often from the ingestion of the organs of other animals dead of the disease. A careful bacteriological examination is here always advisable, especially of any oedematous infiltration about the throat, or in the neighbouring lymphatic glands; often, in pigs dying of anthrax, bacilli may not occur in the blood. Any hæmorrhagic infarction in the spleen of a suspected animal should be carefully investigated. The white rat is much less

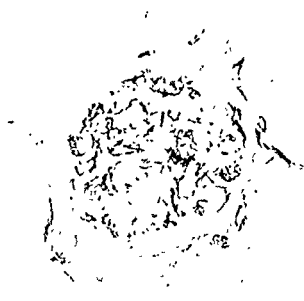


FIG. 63. Section of kidney of guinea-pig dead of anthrax, showing the bacilli in the capillaries, especially of the glomerulus stained by Gram's method and picric acid. $\times 300$

susceptible to the infection than the brown rat. Adult carnivora are also very resistant, and birds and amphibia are in the same position. In the tissues of birds inoculated with spores, the organisms appear to be rapidly killed. The human subject may be said to occupy a medium position between the highly susceptible and the relatively immune animals.

With the less susceptible animals, the bacilli are found in the pathological changes, especially in the tissues of the organs.

Of the less susceptible animals. Instead of the widespread occurrence described above, they may be confined to the point where they first gained access to the body and the lymphatic system in relation to it, or may be only very sparsely scattered in organs such as the spleen (which is often not enlarged), the lungs, or kidneys.

Of the animals commonly used in laboratory work, guinea-pigs and mice are the most susceptible to anthrax, and are generally used for test inoculations. If a small number of anthrax bacilli be injected into the subcutaneous tissue of a guinea-pig, a fatal result follows, usually in two to four

days. *Post mortem*, around the site of inoculation the tissues, owing to intense inflammatory œdema, are swollen and gelatinous in appearance, small hæmorrhages are often present, and on microscopic examination numerous bacilli are seen. The internal organs show congestion and cloudy swelling, with sometimes small hæmorrhages, and their capillaries contain large numbers of bacilli (Fig 63), as has already been described in the case of cattle, the spleen also shows a corresponding condition. Highly susceptible animals may be infected by being fed with spores or by being made to inhale the bacilli or their spores, a general infection rapidly occurring by both methods. Besredka brought forward observations to support the view that the guinea-pig and the rabbit are susceptible only to inoculation of the skin, and he also stated that when the bacilli are injected intravenously or intra-

occurrence of infection are specially favourable when the organisms are inoculated into the skin

A single bacillus taken directly from the spleen of a mouse dead of anthrax septicæmia, produces a fatal infection in twenty-five per cent of mice inoculated subcutaneously (Martos)

The Spread of the Disease in Nature. We have seen that *B anthracis* rarely, if ever, forms spores in the body, and if the bacilli could be confined to the blood and tissues of carcasses of animals dying of the disease, it is likely that anthrax in an epizootic form would be less frequent. For it has been shown by many observers that in the course of the putrefaction of such a carcass the anthrax bacilli rapidly die out, and that after ten days or a fortnight very few remain. But it must be remembered that while still alive an animal is discharging by the bloody excretions from the mouth, nose, and bowel, myriads of bacilli which may rapidly sporulate, and thus arrive at a very resistant stage. These lie on the surface of the ground and are washed off by surface water. It is in the condition of spores that they are dangerous to susceptible animals. In the bacillary stage, if swallowed, they will be killed by the acid gastric contents, but as spores they can pass uninjured through the stomach, and gaining an entrance into the intestine, infect its wall, and ultimately reach, and multiply in, the blood. It is known that in

relatively infrequent, and the disease occurs mostly in sporadic form. Cases are most frequent in the winter and among cattle, and the evidence points to infection by imported food-stuffs, e.g. maize meal, oil-cake, etc. *B anthracis* has been demonstrated in such materials

The Disposal of the Carcasses of Animals dead of Anthrax.¹ It is extremely important that anthrax carcasses should be disposed of in such a way as to prevent their becoming future sources of infection. If anthrax be suspected as the cause of death, no post-mortem examination should be made, but only a small quantity of blood removed from an auricular vein for bacteriological investigation. If such a carcass be now buried six feet below the surface of the earth and with a layer of quicklime not less than 1 foot thick surrounding it both above and below, little danger of infection will be run. The bacilli, being confined within the body, will not sporulate, and will die during the process of putrefaction. The danger of sporulation taking place is, of course, much greater when an animal has died of an unknown disease, which, on post-mortem examination, has proved to be anthrax, but similar measures for burial must be here adopted. In some

¹ The method of disposal of anthrax carcasses and the treatment of infected premises are prescribed in the Anthrax Order of 1928.

cases have been housed, etc., must be washed with a 5 per cent solution of phenol (or an approved substitute). Needless to say, the greatest care must be taken in the case of men who handle the animal or its carcass that they have no wounds on their persons, and that they thoroughly disinfect themselves by washing their hands, etc., in 1 : 1,000 solution of corrosive sublimate or in 1 : 20 lyson. All clothes soiled with blood, etc., from anthrax animals, should be thoroughly boiled or steamed for half an hour before being washed.

Anthrax in the Human Subject. As we have noted, man occupies a middle position in the scale of susceptibility to anthrax. It is always communicated to him from animals directly or indirectly, and usually is seen among those whose trade leads them to handle the carcasses or skins of animals which have died of the disease. It occurs in two principal forms, the main difference between which is due to the site of entrance of the organism into the body. In one, the path of entrance is through cuts or abrasions in the skin, or through the hair follicles. A local condition called a 'malignant pustule' develops, which may lead to a general infection. This variety occurs chiefly among butchers and those who work among hides (foreign ones especially). In Britain the workers of the latter class chiefly liable are the hide-porters and hide-workers in South-East London. Occasionally the disease has been contracted from anthrax spores in shaving-brushes made from the hairs of infected animals. In the other variety of the disease the site of infection is the trachea and bronchi, and here a fatal result almost always follows. The cause is the inhalation of dust or filaments from wool or hair which has been taken from animals dead of the disease, and which has been contaminated with blood or secretions containing the bacilli, these having afterwards formed spores. This variety is often referred to as 'woolsorter's disease', from its occurring in the centres of the woolstapling trade (in England, chiefly in Yorkshire), but it also is found in places where there are hair, brush, or carpet factories.

Malignant Pustule. This usually occurs on the exposed surfaces—the face, hands, forearms, and back, the last being a common site among hide-porters. One to three days after inoculation a small red painful papule appears, soon becoming a vesicle, which may contain clear or blood-stained fluid, it is rapidly surrounded by an area of intense congestion. Central necrosis occurs, and leads to the malignant pustule proper, which in its typical form appears as a black eschar of irregular shape often surrounded by a ring of vesicles, these in turn being surrounded by a congested area. From this pustule as a centre subcutaneous œdema spreads, especially in the direction of the lymphatics, the neighbouring glands are enlarged. Sometimes there may be marked local œdema without a recognisable 'pustule'. There is usually fever with general malaise. On microscopic section of the typical pustule, the central eschar is noticed to be composed of necrosed tissue and degenerating blood cells; the vesicles are formed by the raising of the stratum corneum from the rete Malpighii. The latter is swollen and œdematous, the papillæ being enlarged and flattened out and infiltrated with inflammatory exudation which also extends beneath the centre of the pustule. In the tissue next the eschar commencing necrosis is observed. The subcutaneous tissue is also œdematous, and often infiltrated with leucocytes. The bacilli exist in the periphery of the eschar and in the neighbouring lymphatics, and, to a certain extent, in the vesicles (Fig. 64). They may be very scanty and though found in sections, films and cultures from scrapings may not show them. It is very

important to note that widespread œdema of a limb, enlargement of neighbouring glands, and fever may occur while the bacilli are still confined to the immediate neighbourhood of the pustule. Sometimes the pathological process goes no further, the bacilli gradually die out, the eschar becomes a scab, the inflammation subsides, and recovery takes place. In other cases, however, the œdema spreads, invasion of the blood stream may occur, and the patient dies with, in a modified degree, the pathological changes detailed in regard to the acute disease in cattle. In man the spleen is usually not much enlarged, and the organs generally contain few bacilli.

Woolsorter's disease. The local lesion is usually situated in the lower part of the trachea or in the large bronchi, and is in the form of swollen patches in the mucous membrane, often with hæmorrhage into them—small ulcers may also be seen. The tissues are intensely inflamed, œdematous, and the cellular elements are separated, but there is usually little or no necrosis.



FIG. 100. LUNG IN ANTHRAX.

There is great enlargement and engorgement of the mediastinal and bronchial glands, and hæmorrhagic infiltration of the cellular tissue in the region. There are pleural and pericardial effusions, and hæmorrhagic spots occur beneath the serous membranes. The lungs show great congestion, collapse, and œdema. There may be cutaneous œdema over the chest and neck, with enlargement of glands, and the patient rapidly dies with symptoms of pulmonary embarrassment, and with a varying degree of pyrexia. It is to be noted that in such cases, though numerous bacilli are present in the bronchial lesions, in the lymphatic glands, and affected tissues in the thorax, comparatively few may be present in the various organs, such as the kidney, spleen, etc., and sometimes it may be impossible to find any.

Intestinal anthrax. Infection occasionally takes place through the intestine probably by ingestion of spores as in the case of animals, but this condition is rare. In such cases there occur single or multiple local hæmorrhagic lesions in the intestinal mucous membrane, the central parts of the hæmorrhagic areas tending to be necrotic and yellowish, and there may be a corresponding affection of the mesenteric glands.

A considerable number of cases have been recorded in which *hæmorrhagic meningitis*, associated with the presence of the anthrax bacilli in large numbers, has occurred as a complication of various primary lesions.

Immunity against Anthrax. *Active immunisation.* Having ascertained that there was ground for believing that in cattle one attack of anthrax protected against a second, Pasteur (in the years 1880-82) elaborated a method by which a mild form of the disease could be given to animals, and which rendered harmless a subsequent inoculation with virulent bacilli. He found that the continued growth of anthrax bacilli at 42° to 43° C. caused them to lose their capacity of producing spores, and also gradually to lose their virulence, so that after twenty-four days they could no longer kill either guinea-pigs, rabbits, or sheep. Such cultures constituted his *premier vaccin*, and protected against the subsequent inoculation with bacilli which had been grown for twelve days at the same temperature, and the attenuation of which had therefore not been carried so far. The latter constituted the *deuxième vaccin*. It was further found that sheep thus twice vaccinated now resisted inoculation with a culture which usually would be fatal. After the efficacy of vaccination in this way had been experimentally established on a large scale, the method was employed as a preventive measure and regularly used in France and elsewhere. It is to be noted that the state of active immunity passes off—in a considerable proportion of cases at the end of a year. Koch found also that vaccinated sheep which resisted subcutaneous inoculation might become infected when fed with spores.

In France, during the twelve years 1882-93, 3,296,815 sheep were vaccinated, with a mortality, either after the first or second vaccination, or during the subsequent twelve months, of 0.94 per cent, as contrasted with the ordinary mortality in all the flocks of the districts of 10 per cent. During the same time 438,824 cattle were vaccinated, with a mortality of 0.34 per cent, as contrasted with a probable mortality of 5 per cent if they had been unprotected.

The Pasteurian method of vaccination against anthrax apparently yielded most successful results when first applied, but has been subject to a good deal of criticism in recent times. The vaccines are difficult to standardise, and attenuation at 42° to 43° C. may not proceed in the regular manner originally observed by Pasteur. If their virulence is too high, vaccinated animals may die from the inoculation and even spread the disease; if too low, an effective immunity does not result.

While larger susceptible animals can be immunised by the methods above described, it has been found difficult or often impossible to produce immunity experimentally by this method in the guinea-pig and the rabbit, mainly owing to their susceptibility to a fatal infection when injected subcutaneously with Pasteur's vaccines. Besredka has found that immunity can be developed when inoculation by the skin is employed, either intracutaneous injection or rubbing a culture over a shaved area of skin, the vaccines as used by Pasteur being successively applied, followed by virulent cultures. Grierson (1929) showed that rabbits can be safely and effectively immunised by Besredka's cutaneous method. As a result they can tolerate large doses of virulent organisms injected intravenously. It seems likely, however, that these results depend on the lesser susceptibility of the animal to a fatal infection when the vaccine is introduced into the skin as compared with subcutaneous injection. In the case of a very susceptible animal such as the guinea-pig, the method may fail, however. Following on Besredka's work the intracutaneous method of immunising animals with an anthrax vaccine has been extensively used by Velu; successful results have been

vaccine ' prepared from strains of a standard degree of virulence preserved in glycerol-salt solution. It is injected simultaneously with antiserum, as in the combined method of immunisation referred to below. Mixtures of saponin or digitonin with suspensions of virulent anthrax bacilli or spores when injected subcutaneously into rabbits and large animals cause no serious effects and a single dose leads to immunity (Hruška).

Anti-anthrax sera have been prepared from highly immunised animals, and such sera have been found to possess protective and, to a certain extent, curative properties. In their preparation it is necessary to use living cultures of the bacillus; attenuated cultures are usually employed first, and later virulent cultures. The method has been combined with the use of an antiserum obtained from an animal already immunised—that is, a combination of active and passive immunisation is carried out. The two best known sera are those of Sclavo and Sobernheim. The former, who found the ass the most suitable animal for immunisation, published favourable results obtained in the treatment of malignant pustule. Out of 164 cases there were only ten deaths, this representing about a fourth of the ordinary mortality in Italy. The serum is administered by intramuscular or by intravenous injection. Sabolotny has reported favourable results in very severe human cases with bacilli present in the blood, who received the serum intravenously. A large initial intravenous dose of serum should be given (100 to 250 c c) and repeated if necessary at intervals of twenty-four hours, one to six injections being administered in all. In this way severe cases have been treated successfully even when lung lesions and septicæmia were present (see Lucchesi, and Gold). An important additional point in treatment is to avoid any interference with the local lesion; the part should be kept completely at rest.

Combined immunisation. Sobernheim's serum has been used for protective purposes in animals, the antiserum being injected at the same time as Pasteur's second vaccine but at a separate site. This method has been widely used in Europe and South America, and it is claimed that it has advantages over Pasteur's, especially in that its application is safer and simpler, one operation instead of two being sufficient.

Mechanism of immunity to anthrax. While anti-anthrax sera produced by the administration of living organisms have markedly preventive properties, it does not seem possible to explain their action by antibodies which can be demonstrated *in vitro*. Sobernheim and others were unable to detect in such sera immune bactericidal properties, and Sclavo came to the conclusion that in the action of the serum substances of the nature of antibody and complement are not concerned. Opsonic action has been put forward in explanation, but this has not been accepted by some writers. It is a striking fact that from the blood and tissues of immunised animals which have tolerated inoculation with the organisms, living virulent anthrax bacilli can be recovered even after a week. Anti-anthrax serum may contain a specific precipitin, agglutinin, and complement-fixing antibody (*vide infra*), but this, of course, does not explain the beneficial effect of the serum. Bail considered that the protective action of an immune serum is due to its containing antiaggressins. It has been suggested that the capsular substance acts as an aggressin by combining with opsonin and so protecting the organism from phagocytosis, on this basis the protective action of immune serum might be regarded as due to its high content of antibodies which are capable of reacting specifically with the capsular antigen. In this connection Tomcsik and others have shown that the capsular protein and the somatic polysaccharide of the anthrax bacillus possess antigenic or haptenic properties, each of which is distinct, although the same capsular protein occurs also

in other members of the biological group to which the organism belongs (*vide infra*). Antisera developed by injecting the capsulate bacilli, after being absorbed with a non-capsulate anthrax culture, act as a pure antibody to the capsular protein. The latter antibody gives precipitation with high dilutions of the soluble protein and also the complement-fixation reaction; it produces changes in the capsules which can be detected by the microscope, and agglutinates the capsulate bacilli. By the use of capsular antibody it has been shown that the capsular material formed by the bacilli in the tissues after inoculation with a virulent non-capsulate culture, is serologically identical with that formed *in vitro* by strains which produce a capsule in culture. The polysaccharide appears to be specific for the anthrax bacillus.

The problem of the nature of acquired immunity to anthrax is one of considerable complexity, as illustrated by the observations of Tomcsik and Ivánovics. While immunity in one species (mouse) is related to the antibodies reacting with the capsular substance, immunity in another (rabbit) does not depend on these antibodies. This is shown by the fact that rabbits injected with killed cultures of the capsulate bacilli possess capsular antibody, and their serum confers passive immunity on mice against infection with virulent organisms, on the other hand, the rabbits themselves do not possess a solid immunity. Again, the serum of rabbits which have been rendered actively immune by injections of the sterilised cedema fluid of infected rabbits, does not confer passive immunity on mice; neither antibodies to the capsular substance nor the somatic polysaccharide can be demonstrated in such immune serum. Stamatin has also pointed out that there is no relation between immunising power and capsulation and that living bacilli which are non-capsulate are still capable of inciting a high degree of immunity in rabbits. However, the part played by the somatic (polysaccharide) antigen in developing such immunity in the rabbit is not clear. The subject requires further investigation and at present no definite conclusions can be drawn as to the true nature of anti-anthrax immunity. The nature of the normal resistance of certain apimal species is also obscure.

Ascoli's thermo-precipitin reaction, etc. Ascoli's reaction depends on the observation that certain anthrax immune sera produce a precipitin reaction with the products of *B. anthracis*. The suspected blood or tissue is boiled for a few minutes in 5 to 10 volumes of normal saline which may be acidified by one part per thousand of acetic acid, the fluid is cooled and filtered through paper or asbestos so as to obtain a clear filtrate, a little of this is then run on to the top of the serum, and a white ring should

from an undoubted anthrax case, as only a certain small proportion of animals give the reaction. The reaction seems to depend on an effect produced between the serum and substances derived from the bacilli, as it is most marked with tissues containing numerous organisms. It can be obtained with material which has been kept for six months, and numerous controls made with tissues of animals dying from other diseases are stated to have given negative results. A method of obtaining active antisera for the precipitin test has been described by Rosenberg and Romanow.

Bacteriological Diagnosis. *Microscopic examination.* In a case of suspected malignant pustule, film preparations should be made from the fluid in the vesicles or from the surface exudate and stained by Gram's method. In this way preliminary evidence may be obtained. Occasionally bacilli are so scanty that both film preparations made from different parts and even cultures may give negative results. Care ought to be taken in manipulating a pustule, as, otherwise, the diffusion of the bacilli into the surrounding tissues may be aided. The examination of the blood in cases of anthrax in man usually gives negative results, with the exception of very

severe cases, when a few bacilli may be found in the blood shortly before death.

Cultivation. The material should be stroked on agar plates. After twenty-four hours at 37° C colonies of *B anthracis* will appear, and, as has been described, can be readily recognised by means of a hand-lens.

While the isolation of the *B. anthracis* from fresh material is usually easy, great difficulty may be encountered where the organism is to be sought for in an animal which has been dead for twenty-four hours or longer, as the bacilli rapidly die or are associated with putrefactive organisms. Where cultures cannot be made at once from blood or tissue fluids, the material should be preserved by smearing it on unglazed earthenware and allowing it to dry.

Animal inoculation test This method is used for confirming the identity of an organism suspected to be the anthrax bacillus from the microscopic and cultural examination. Guinea-pigs or mice are injected, usually subcutaneously, with material from the pustule or the pure culture isolated. In the case of hair or hides the suspected material is soaked in sterile water and well teased out or scraped. After the mixture has stood for about an hour, the fluid is decanted and centrifuged, and the sediment is suspended in 1 c.c. of sterile water and heated at 65° C for five minutes. It is then injected subcutaneously into one or two guinea-pigs (mice are less satisfactory). If anthrax bacilli are present, the animals usually die within four days, with the changes in internal organs already described, and the bacilli can be demonstrated in the heart blood. *The identification of an organism as the anthrax bacillus cannot be said to be substantiated till its pathogenicity has been proved.* When death occurs from a mixed infection with anaerobes, the anthrax bacillus may be recovered *post mortem* by making aerobic plate cultures from the peritoneal fluid. Another method is to inoculate contaminated material into the scarified skin, as the anthrax bacillus is more likely to cause infection by this route than are the other pathogens which may be present.

ORGANISMS BIOLOGICALLY ALLIED TO THE ANTHRAX BACILLUS

bacillus is the only actively pathogenic representative of the group. The others are saprophytes, occurring in soil, dust, water, etc. Spores of these organisms are practically ubiquitous and frequently contaminate culture medium, especially when it is exposed to air. Some of them so closely resemble *B anthracis* that, apart from animal inoculation tests, they may be easily mistaken for it. Thus morphological and cultural characters almost identical with those of the anthrax bacillus may be presented by certain of these organisms though in some cases motility in culture clearly distinguishes them from *B anthracis*. This is well exemplified by the so-called *B anthracoides*. Inoculation of a mouse or guinea-pig, to which *B anthracis* is extremely virulent, serves as the essential method of differentiation. It is noteworthy, however, that certain organisms of this type (e.g. *B anthracoides*) are not entirely devoid of pathogenicity under experimental conditions, and may on subcutaneous inoculation produce a lethal effect in mice and guinea-pigs if large doses of cultures are used (Grierson, 1928). Even a septicæmic condition may result, as in anthrax, but the organisms in the blood and spleen fail to show McFadyean's reaction. A small dose of a recently isolated anthrax bacillus is, of course, sufficient to cause death in a susceptible animal.

Classical types belonging to this group are *B. mycoides*, *B. subtilis*, *B. mesentericus* (*vulgatus*). *B. mycoides* is very similar in morphology to *B. anthracis* (though the spore is often excentric in position), but its colonies on nutrient agar differ from those of the latter in exhibiting a feathery or spiked appearance. *B. subtilis* also resembles the anthrax bacillus in morphology, but the ends are often rounded, there is less tendency to chain formation, and in young cultures the bacilli show active motility and peritrichous flagella. The spores are small (0.5 to 0.8μ) and may be central or excentric. This organism is strictly aerobic and grows well at low temperatures, though the optimum is 37°C . A stroke inoculation on culture medium produces an abundant dry, opaque, adherent, greyish-white spreading layer of growth without the characteristic wavy or wreathed margin of *B. anthracis* cultures. A potato culture consists at first of a moist layer of growth, but later assumes a dry mealy appearance. According to Axenfeld, *B. subtilis* may produce infection of the eye with resulting iridocyclitis and panophthalmitis. *B. mesentericus* closely resembles *B. subtilis*, and is characterised by the marked wrinkling and folding of the growths on artificial media and brown coloration of older cultures. Another member of this group described in the early literature of bacteriology, *B. megatherium*, produces very large ovoid spores and shows only sluggish motility, flagella being not always seen, growths are smooth, butyrous, and cream-coloured, becoming in old cultures yellowish-red.

A very large number of species or types have been described in the group (see Bergey) and a considerable variety of characters are represented within the standard features of the genus *Bacillus* to which these organisms are assigned in the new classification and nomenclature. It may be noted that while some species grow best from 30° to 37°C ., most have a lower optimum, e.g. 20°C .; on the other hand some are thermophilic (optimum temperature, 55°C). Many species yield pigmented growths and some produce a black pigment. Proteolytic activity is a common feature of the group and some species are also saccharolytic.

CHAPTER XI

THE BACILLI OF GLANDERS AND MELIOIDOSIS

THE bacillus of glanders, originally designated *Bacillus mallei*, was discovered by Löffler and Schütz, the announcement being made in 1882. They not only obtained pure cultures of this organism from the tissues in the disease, but by experiments on horses and other animals conclusively established its causal relationship. These have been fully confirmed. The same organism was also cultivated from the disease in the human subject, first by Weichselbaum in 1885, who obtained it from the pustules in a case of acute glanders, and by inoculation of animals obtained results similar to those of Löffler and Schütz.

Glanders chiefly affects equines—horses, mules, and asses. Cattle and pigs, on the other hand, are quite immune, while goats and sheep occupy an intermediate position, the former being rather more susceptible and occasionally suffering from the natural disease, as do also camels. Glanders occurs in some of the carnivora—cats, and lions and tigers in

tion with the allied organism of melioidosis occurs naturally in these animals. While prevalent at one time in this country, particularly among horses, glanders has been eradicated as a result of diagnosis of early and latent infections by means of mallein, and slaughter of infected animals as laid down by the Glanders or Farcy Order (1907).

Glanders may occur also in man as the result of direct inoculation of some wound of the skin or other part by means of the discharges or diseased tissues of an affected animal, and hence has been commonest among grooms and others whose work brings them into contact with horses, even among them it has been an uncommon disease.

In horses the lesions are of two types, to which the names 'glanders' proper and 'farcy' have been given though both may exist together. In glanders proper, the septum nasi and adjacent parts are chiefly affected, there occurring in the mucous membrane nodules which are at first firm and of somewhat translucent grey appearance.

glands in the neck, mediastinum, etc., and there may be in the lungs, spleen, liver, etc., nodules of the size of a pea or larger, of greyish or yellow tint, firm or somewhat softened in the centre, and often surrounded by a congested zone. The term 'farcy' is applied to the affection of the superficial lymphatic vessels and glands, which is specially seen

secondary nodules may occur in internal organs and the nasal mucous membrane. The disease is often present in a 'latent form', and its presence can only be detected by the mallein allergic test.

In man the disease is met with in two forms, an acute and a chronic—though intermediate forms also occur, and chronic cases may take on the characters of the acute disease. The site of inoculation is usually on the hand or arm—by means of some

scratch or abrasion, or possibly by infection along a hair follicle—sometimes on the face, and occasionally on the mucous membrane of the mouth, nose, or eye. In the acute form these changes at the site of inoculation inflammatory swelling, attended usually with the part also become inflamed, the these local changes are soon followed local or widespread eruption on the surface of the body, at first papular and afterwards pustular, and later there may form

often foul, discharge. The ulceration spreads deeply as well as superficially, and the thickened lymphatics also have a great tendency to ulcerate, though the lymphatic



FIG. 65. Glanders bacilli—several contained within leucocytes—from peritoneal exudate in a guinea-pig. Stained with dilute carbol-fuchsin $\times 1,000$

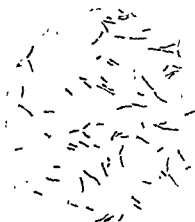


FIG. 66. Glanders bacilli, from a pure culture on glycerol agar. Stained with carbol-fuchsin and partially decolorised to show segmentation of protoplasm $\times 1,000$.

system is not so prominently affected as in the horse. Deposits may form in the subcutaneous tissue and muscles, and the mucous membrane may become affected. The disease may run a very chronic course, lasting for months or even years, and recovery may occur; on the other hand, such a case may at any time take on the characters of the acute form of the disease and rapidly become fatal. Even when there is apparent recovery recurrence may occur.

While spontaneous recovery from the infection may occur both in man and animals, no method of preventive immunisation is known.

BACILLUS MALLEI

(*MALLEOMYCES*, OR *PFEIFFERELLA*, MALLEI)

MICROSCOPIC CHARACTERS The bacilli are rod-shaped, straight or slightly curved, with rounded ends (Fig. 65), and about 2 to 3μ by 0.4μ . They show, however, considerable variations in size and in appearance, and their protoplasm is often broken up into a number of deeply stained portions with unstained intervals between. These characters are seen both in the tissues and in cultures, but, as in the case of many organisms, irregularities in form and size are more pronounced in cultures (Fig. 66). Filamentous forms, 8 to 12μ in length, are sometimes met with and branching has been described. The organism is non-motile and does not form spores.

In the tissues the bacilli usually occur irregularly scattered among the cellular elements, a few may be contained within leucocytes and connective tissue cells, but the position of most is extracellular. They are most abundant in the acute lesions, in which they may be found in considerable numbers; but in the chronic nodules, especially when softening has taken place, they are few in number, and it may be impossible to find any in sections.

The glanders bacillus stains with simple watery solutions of the basic stains, but somewhat faintly (better when an alkali or a mordant, such as carbolic acid, is added), and even if deeply stained it readily loses the colour when a decolorising agent such as alcohol is applied. It is Gram-negative. In film preparations from fresh glanders nodules the bacilli can be readily demonstrated by staining with carbol-thionin or dilute carbol-fuchsin. In sections, the best results are obtained with carbol-thionin and by dehydrating by aniline-xylol.

McFadyean recommended that after sections have been stained in Löffler's methylene blue and slightly decolorised in weak acetic acid, they should be treated for fifteen minutes with a saturated solution of tannic acid, thereafter they are washed thoroughly in water, and as a contrast-stain a 1 per cent solution of acid fuchsin may be applied for half a minute, they are then dehydrated, cleared, and mounted.

CULTURAL CHARACTERS The glanders bacillus is an aerobe and facultative anaerobe and grows readily on most of the ordinary media, the optimum temperature being 35° to 38° C. A certain amount of growth occurs down to 21° C.

On *agar*, in stroke cultures, growth appears along the line as a uniform streak of greyish-white colour and somewhat transparent appearance, with moist-looking surface, and when touched with a wire is found to be of rather slimy consistence. Later it spreads laterally for some distance, and the layer becomes of slightly brownish tint. Separate colonies are small disks, about 1 mm in diameter, with regular borders and have the same physical characters as the stroke culture. In subcultures at the body temperature colonies are visible within twenty-four hours, but when fresh cultures are made from the tissues there may be no visible growth for two or three days. The addition of 3 to 4 per cent. glycerol enhances growth.

On *serum media* the growth is somewhat similar but more transparent. Serum media are much more suitable for cultivating from the tissues than agar media.

In *broth*, growth forms at first a uniform turbidity, but soon settles to the bottom, and after a few days forms a fairly thick flocculent deposit of slimy and somewhat tenacious consistence.

On *potato* (provided the reaction is not too acid), at 30° to 37° C. the glanders bacillus flourishes well and produces a characteristic appearance, incubation at the higher temperature, however, being advisable. Growth

ed a transparent layer

On subsequent days

and more opaque, till

e tint. Potato is also

a suitable medium for starting cultures from the tissues, in this case minute transparent colonies become visible on the third day, and afterwards present the appearances just described.

Rough and smooth *variants* have been described.

BIOCHEMICAL REACTIONS are not pronounced. Acid formation from glucose has been found, but this is not constant. Indole is not produced and gelatin is not usually liquefied but the organism does not grow well at the low temperatures suitable for gelatin cultures.

VIABILITY. The glanders bacillus is not killed at once by natural drying, but usually loses its vitality after fourteen days in the dry condition, though sometimes it lives longer. It is not quickly destroyed by putrefaction, as it has been found to be still active after remaining two or three weeks in putrefying fluids. It has comparatively feeble resistance to heat and antiseptics. Löffler found that it was killed in ten minutes in a fluid kept at 55° C., and in from two to three minutes by a 5 per cent. solution of carbolic acid. Primary cultures tend to die rapidly, but when adapted to laboratory growth the bacilli may survive for two months.

Experimental Inoculation. In horses, subcutaneous injection of the glanders bacillus in pure culture reproduces all the essential features of the disease. This fact was established at a comparatively early date by Löffler and Schutz, who successfully inoculated horses in this way, the cultures used having been grown for several passages outside the body. The ass and mule are even more susceptible than the horse, the disease in the former running a more rapid course, but with similar lesions. The ass can be readily infected by simple scarification and inoculation with glanders secretion, etc. (Nocard). Infection by feeding is also successful.

Of small animals, field-mice and guinea-pigs are the most susceptible; on the other hand, white mice are more resistant and after inoculation with pus containing *B. mallei* alone they live for five or six weeks (Sabotny). In field-mice, subcutaneous inoculation is followed by a very rapid disease, usually leading to death within eight days, the organisms becoming generalised and producing numerous minute nodules, especially in the spleen, lungs, and liver. In the guinea-pig the disease is less acute. At the site of inoculation an inflammatory swelling forms, which soon softens and breaks down, leading to the formation of an irregular crateriform ulcer with indurated margins. The lymphatic vessels become infiltrated, and the corresponding lymphatic glands become enlarged to the size of peas or small nuts, softened, and semi-purulent. The animal sometimes dies in two or three weeks, sometimes not till later. Secondary nodules, in varying numbers in different cases, may be present in the spleen, lungs, bones, nasal mucous membrane, testicles, etc., sometimes a few nodules are found in the spleen alone. Intraperitoneal injection in the male guinea-pig is followed, as pointed out by Straus, by a very rapid and semi-purulent affection of the tunica vaginalis, shown during life by great swelling and redness of the testicles, which changes may be noticeable in two or three days, or even earlier. This method of inoculation has been found of service for purposes of cultivation and diagnosis, however, strains of low virulence may fail to produce the characteristic results, while certain other organisms may cause the same lesions. Rabbits are less susceptible than guinea-pigs, and the effect of subcutaneous inoculation is somewhat uncertain. Accidental inoculation of the human subject with pure cultures of the bacillus is a considerable risk among those handling them and has in more than one instance been followed by the acute form of the disease and a fatal result.

Pathogenic Action and Toxin. Though glanders has been generally classified among the infective granulomata, the glanders bacillus causes a more rapid and more marked inflammatory reaction than the tubercle bacillus, there is more leucocytic infiltration and less proliferative change. Thus the centre of an early glanders nodule shows a dense aggregation of leucocytes, most of which are polymorphonuclear. Further, the inflammatory change may be followed by suppurative softening of the tissue, especially in certain situations, such as the subcutaneous tissue and lymphatic glands. The nodules, therefore, in glanders, as Baumgarten stated, occupy an

intermediate position between miliary abscesses and tubercles. The diffuse coagulative necrosis and caseation which are so common in tubercle, do not occur to the same degree in glanders, and typical giant-cells are not formed. The tendency to spread by the lymphatics is always a well-marked feature, and when the bacilli gain entrance to the blood stream they soon settle in the various tissues and organs. Even in acute cases it is usually impossible to detect the bacilli in the circulating blood, though sometimes they have been found. It is an interesting fact, shown by observations of the disease both in the human subject and in the horse, as well as by experiments on guinea-pigs, that the mucous membrane of the nose may become infected by means of the blood stream—another example of the tendency of organisms to settle in special sites.

The *toxin* of the bacillus is an endotoxin. Thus, dead cultures may cause marked general toxic effects in laboratory animals and even fatal results. This endotoxin would account for the severe constitutional symptoms of the disease.

Spread of Infection. Glanders usually spreads from a diseased animal by direct contagion with the discharge from the nose or from the sores, etc. There is no evidence that in man the disease is produced by inhalation of the bacilli in the dried condition. Some authorities consider that pulmonary glanders may be produced in this way in the horse, while others maintain that in all cases there is first a lesion of the nasal mucous membrane or of the skin surface, and that the lung is affected secondarily. Babés, however, found that the disease could be readily produced in susceptible animals by exposing them to an atmosphere in which cultures of the bacillus had been pulverised. He also found that inoculation of the skin with vaseline containing the bacilli might produce the disease, the bacilli in this case entering along the hair follicles.

Allergy : Mallein and its Preparation. In infections with *B. mallei* super-sensitiveness develops to products of the bacillus ('mallein') analogous to that of tuberculous subjects to tuberculin. Mallein is obtained from cultures of the glanders bacillus and, like tuberculin, is really a mixture comprising substances in the bodies of the bacilli, and their soluble products, along with substances derived from the culture medium. It is usually prepared from cultures in glycerol broth. Such cultures, after being allowed to grow for three or four weeks, are sterilised by heat either in the autoclave at 115° C. or by steaming at 100° C. and then filtered through paper. The filtrate constitutes dilute fluid mallein; it may then be concentrated to one-tenth of its volume by evaporation (crude mallein) or it may be preserved in the dilute form with the addition of 0.5 per cent. carbolic acid. Of dilute mallein (or of the concentrated product diluted 1 : 10) 1 c.c. is usually the dose for a horse. Foth has prepared a dried form of mallein by mixing the filtrate of a broth culture, evaporated to one-tenth of its bulk, with twenty-five or thirty times its volume of alcohol. A white precipitate is formed, which is dried over calcium chloride and then under diminished pressure. A dose of this dry mallein is 0.05 to 0.07 grm. dissolved in sterile water before use.

The use of mallein as a means of diagnosis. In using mallein for the diagnosis of glanders the temperature of the animal ought to be observed for some hours beforehand, and after subcutaneous injection of a suitable dose it is taken at definite intervals—at the sixth, tenth, fourteenth and eighteenth hours and on the next day. Here both the local reaction and the temperature are of importance. In an infected animal at the site of injection there is a somewhat tender local swelling which reaches a diameter of 5 inches at least the maximum size not being attained until twenty-four hours afterwards. The temperature rises 1.5 to 2° C., or more, the maximum generally occurring in from eight to sixteen hours. If the temperature never rises as much as 1.5°, the reaction

is considered doubtful. In the negative reaction given by an animal free from glanders, the rise of temperature does not usually exceed 1° , the local swelling reaches the diameter of 3 inches at most, and has much diminished at the end of twenty-four hours. In the case of dried mallein, local reaction is less marked. Also 0.2 c.c. crude mallein instilled into the conjunctival sac, gives in infected animals a reaction corresponding to the ophthalmic tuberculin reaction in tuberculosis. Reactions may be obtained in glanders by cutaneous or intracutaneous applications of mallein. The intradermal-palpebral test has been much used; 0.1 c.c. of a 25 per cent. dilution in saline of crude mallein

ld. Veterinary authorities are practically unanimous as to the great value of the mallein test as a means of diagnosis. But animals at an advanced stage of the disease may fail to react.

Serum Reactions. Shortly after the discovery of the agglutination reaction in typhoid fever, McFadyean found that the serum of infected horses

glanders bacillus, and this test has
the disease. The culture used for
satisfactory by previous trial. The

suspension is prepared from young glycerol agar cultures and is usually heated at 65°C for one hour to kill the organisms and render it safe for the manipulations involved in the test. Serum dilutions from 1 in 100 to 1 in 4,000 may be tested and after incubation the tubes are allowed to stand overnight at room temperature before the results are read. Normal horse serum, however, may contain agglutinins for the glanders bacillus (see Lovell). Wilson has found titres over 1 in 1,500 with sensitive strains. Accordingly natural agglutination must be excluded by careful quantitative tests. A titre of over 1 in 1,000 is highly suggestive of the presence of glanders. The fixation of complement test is also applicable in the case of glanders, and this has given valuable results in the hands of various observers. A precipitin reaction may be obtained on the addition of mallein or an extract of the glanders bacillus to the serum of an infected animal. These reactions, which depend on the presence of antibodies in the blood in glanders, form important auxiliaries to the method of diagnosis by means of mallein. Different serological races of *B. mallei* have been described.

Bacteriological Diagnosis. Microscopic examination in a case of suspected glanders will at most reveal the presence of bacilli corresponding in their characters to the glanders bacillus. An exact diagnosis cannot be made by this method. Cultures may be obtained by making successive strokes on coagulated serum or glycerol agar, and incubating at 37°C . The colonies of the glanders bacillus do not appear till two or three days afterwards. This method may fail unless a considerable number of glanders bacilli are present. The most certain method, however, is by inoculation of a guinea-pig. After intraperitoneal injection in a male guinea-pig the characteristic Straus reaction is rapidly produced. If, however, there have been other organisms present, the animal may die of septic peritonitis, though even in such a case the glanders bacillus will be found to be more numerous in the tunica vaginalis, and may be cultivated from this situation. Where there is mixed infection, however, it is advisable first of all to inoculate a guinea-pig subcutaneously and then cultivate the organisms from the enlarged lymph glands. The application of mallein in diagnosis of the disease in the human subject is not justifiable. There is a certain risk that it may lead to the lesions assuming a more acute character; moreover, culture and inoculation tests are generally available. In the case of horses, etc., a diagnosis will, however, be much more easily and rapidly effected by means of mallein, or by one of the serum reactions described above. In some cases of acute glanders in the

human subject the bacillus has been obtained in cultures from the blood during life

BACILLUS WHITMORI (OF MELIOIDOSIS)

(*MALLEOMYCES*, OR *PFEIFFERELLA*, *PSEUDOMALLEI*)

Melioidosis, which resembles glanders closely, was first observed by Whitmore in Rangoon, and its main features and the causal organism were described by him in 1913. He cultivated it from 38 cases and gave an account of its characters. The disease was also found by Stanton and Fletcher to occur in the Malay States, and they have shown that it is naturally an epizootic among small rodents—rabbits, guinea-pigs, and rats, cats and dogs also are affected. It is believed that infection in man usually occurs by way of the alimentary tract, probably from ingestion of food contaminated with the bacilli from the faeces of infected rodents. The clinical features of the disease in the human subject are very similar to those of glanders, though it tends to be, on the whole, rather more acute than the latter. The pathological changes likewise are of similar nature, a prominent feature being the occurrence of caseous or semi-purulent areas in the lungs with marked congestion around them, the histological changes also have been found to be practically identical in the two diseases. *B. whitmori* in its morphological characters and staining reactions resembles *B. mallei*. It differs from the latter, however, in being actively motile, in growing on gelatin at 20° C. in which it produces liquefaction—as it does also on solidified serum—and in forming a pellicle in broth. The growth on glycerol agar is of two types, a corrugated—the commoner type—and a mucoid. The latter type produces on potato a growth similar to that of the glanders bacillus. In culture media, acid (but no gas) is formed slowly from glucose, lactose, saccharose, and mannitol, indole is not produced. It is a fairly resistant organism outside the body. Susceptible animals can be infected by scarification, by feeding, or by the simple application of cultures to the nasal mucosa. A characteristic feature in the infected animals is a discharge from the nose and eyes, and *post mortem* numerous small nodules are found in the internal organs. With small doses of the organisms injected intraperitoneally in the guinea-pig the Straus reaction (*vide supra*) develops. Apparently the disease very rarely occurs in the horse, and Stanton and Fletcher have found that this animal is resistant to inoculation. These observers have found that *B. mallei*, in fact, a strain of *B. mallei*, found that a case of chronic

CHAPTER XII

BACILLUS PYOCYANEUS, BACILLUS PROTEUS, AND BACILLUS FÆCALIS ALKALIGENES

BACILLUS PYOCYANEUS (*PSEUDOMONAS ÆRUGINOSA*)

This organism is commonly found in various suppurative conditions along with other pyogenic organisms. It is characterised by the production of a blue-green pigment

MICROSCOPIC CHARACTERS. It occurs as straight rod-shaped forms, 1.5 to 3 μ in length and about 0.5 μ in thickness but varying considerably in length (Fig. 67); occasionally two or three are found attached end to end. It is actively motile, possessing one to three terminal flagella, and does not form spores. It stains readily with the ordinary basic stains, but is decolorised by Gram's method.



FIG 67. *Bacillus pyocyaneus*; young culture on agar
Stained with dilute carbol-fuchsin.
 $\times 1,000$

CULTURAL CHARACTERS It grows readily under aerobic conditions on all the ordinary media at room temperature, the cultures being distinguished by the formation of a blue-green pigment. Growth is inhibited under anaerobic conditions. The optimum temperature is about 37° C and the temperature range for growth is from about 5° to 42° C. On agar the growth forms an abundant slimy greyish layer which afterwards becomes dark green, and a

bright green colour diffuses through the whole substance of the medium. The colonies are circular, slightly convex, about 2 to 3 mm. in diameter after twenty-four to forty-eight hours, with a smooth surface and moist appearance and show the same pigmentation as the confluent growth; after several days incubation they tend to enlarge and show a thin transparent border with an irregular outline. In stab cultures in gelatin a greyish line appears in twenty-four hours, and at its upper part a small cup of liquefaction forms within forty-eight hours. At this time a slightly greenish tint is seen in the superficial part of the gelatin. The liquefaction extends rapidly, the fluid portion being turbid and showing masses of growth at its lower part. The green colour becomes more marked, and diffuses through the gelatin. Ultimately liquefaction reaches the wall of the tube. In plate cultures the colonies appear as minute whitish points, those on the surface being the larger. Under a low power of the microscope they have a brownish yellow colour and show a nodulated surface, the superficial colonies being thinner and larger. Liquefaction soon occurs, the colonies on the surface forming shallow cups with small irregular masses of growth at the bottom, the deep colonies small spheres of liquefaction. Around the colonies the characteristic green colour appears. On potato the growth is an abundant brown layer resembling that

of the glanders bacillus (*q.v.*). Under anaerobic conditions the green pigment becomes reduced to a colourless 'leuco' compound, but the colour returns on oxygenation.

BIOCHEMICAL REACTIONS. Glucose is fermented with acid but no gas production. Indole is not formed. Ammonia is produced and there is marked proteolytic action, *e.g.* liquefaction of gelatin, coagulated serum, and peptonisation of milk.

From the cultures there can be extracted by chloroform a blue-green pigment, *pyocyanin*, which crystallises in the form of long, delicate blue-green needles; it is also soluble in water. On the addition of a weak acid its colour changes to red. A yellowish-green fluorescent pigment, *fluorescin*, is also produced, which is soluble in water but insoluble in chloroform. When continuously cultivated in the laboratory the organism may lose the power of forming pyocyanin and on plating old laboratory cultures it may be found that only some colonies show the typical blue-green colour, the others having the yellowish or yellow-green colour of fluorescin.

The chemical characters of pyocyanin have been studied by Wrede and others. Its empirical formula is $C_{22}H_{16}N_4O_2$. It is a phenazine derivative. Along with acids, red-coloured salts are formed. Pyocyanin acts as a reversible oxidation-reduction system and as a respiratory catalyst. For the synthesis of fluorescin the organism requires magnesium phosphate and sulphate (Turfitt). This pigment is oxidisable, becoming converted to a yellowish-brown colour, its empirical formula is $C_4H_5O_2N$.

It has been long known that *B. pyocyaneus* is antibiotic towards certain other bacteria, *e.g.* *B. anthracis*, and this has been attributed to a lytic enzyme, 'pyocyanase', demonstrable in filtrates of broth cultures. Recent work has shown that the antibiotic action is due to the pigments of the organism, *e.g.* pyocyanin, the most active is a yellow pigment α -oxyphenazine, which can be separated in the crystalline state from old cultures (see Schoental).

Experimental Inoculation. *Bacillus pyocyaneus* has distinct pathogenic action in certain animals. Subcutaneous injection of small doses in rabbits may produce a local suppuration, but if the dose be large, spreading hæmorrhagic œdema results, which may be attended by septicæmia. Intravenous injection may produce, according to the dose, rapid septicæmia with nephritis, or sometimes a more chronic condition of wasting attended by albuminuria.

Pathogenicity in the Human Subject. This organism occurs naturally in the intestinal canal and is often found on the skin. It is also met with in water, sewage, and decaying organic matter. Though its pathogenicity is relatively low it is a common secondary infecting organism, *e.g.* associated

intestinal irritation, pustular or petechial eruptions in the skin, and general marasmus. It sometimes occurs in cystitis and pyelitis, and in otitis, usually associated with another organism.

***Bacillus fluorescens* (*Pseudomonas fluorescens*)** This is a saprophytic organism which is found in water, sewage, soil, and decomposing organic matter. Certain plants may be attacked by it, *e.g.* tomatoes, carrots, etc.,

of pyocyanin, though fluorescin is produced. Liquefaction of gelatin may be absent. It is practically non-pathogenic on experimental inoculation of animals.

BACILLUS PROTEUS (PROTEUS VULGARIS)

The term *Proteus* has been applied to a group of bacteria, of which several varieties have been described, e.g. *inlgaris*, *mirabilis*, *zenkeri*, *capsulatus*, the so-called *Urobacillus septicus* is also a variety. Organisms of this group are widely distributed in nature, occurring in the intestinal tract of man and animals, manured soil, sewage, water, and decomposing organic matter; they may also be found on the skin. They are characterised by their pleomorphism, hence the name, and by their rapid liquefaction of gelatin.

MICROSCOPIC CHARACTERS. *B. proteus* is a small bacillus, 1 to 3 μ by 0.5 μ , straight or slightly curved, but coccoid and filamentous forms also occur, and a marked tendency to involution forms is to be noted. It is actively motile, and possesses numerous lateral flagella; it does not form spores. It stains readily with basic dyes and is Gram-negative.

CULTURAL CHARACTERS It grows as an aerobe and facultative anaerobe on all the ordinary media at room temperature, but best between 34° and 37° C. The temperature range for growth is from 10° to 43° C. On an agar slope the organism forms a moist translucent layer, which extends over the whole surface of the medium, in this way the bacillus can readily be separated from other organisms present along with it, but in mixed culture other organisms are difficult to separate from *B. proteus* owing to its spreading growth. This type of organism is therefore spoken of as a 'spreader'; the same spreading growth is noted in plate cultures but is not invariably present. In a gelatin stab culture liquefaction appears within twenty-four hours; it develops rapidly in the form of a crater, and ultimately the whole medium undergoes stratiform liquefaction and presents a turbid appearance. In gelatin plates the characters are somewhat peculiar, especially when 5 per cent. gelatin is used. The colonies are at first small spheres with granular centres and peripheral radiations extending into the medium; liquefaction soon follows, and from the superficial colonies offshoots extend over the medium in tendril-like fashion, these being composed of bacilli in chains placed side by side. Groups of bacilli may also become separate, move over the surface of the medium, and form growths at a distance—the so-called 'swarm-colonies'. On *potato* it forms a slimy film with a greyish-brown colour. It coagulates *milk* (without acid reaction) and later digests the casein. Coagulated serum is liquefied by some strains. On *blood agar* growths produce hæmolysis and a brown discoloration. A hæmolysin for rabbits' corpuscles has been demonstrated.

When *B. proteus* occurs in mixed culture with other pathogenic organisms, e.g. streptococci, its spread on the surface of the medium makes the recognition of these organisms difficult and their isolation almost impossible. Various methods have been used for preventing spread of *B. proteus*, so that its colonies remain discrete. A 1 : 8,000 concentration of sodium lauryl sulphate (reckoned on the basis of content of the pure substance) or 1 : 1,000 'Dispersol LN' (I.C.I.) in agar have this effect while permitting growth of staphylococci, streptococci, and the Gram-negative intestinal pathogens (Lominski and Lendrum). For blood agar add first to the melted agar Dispersol LN to a concentration of 1 : 500 and mix, then add 10 per cent. oxalated horse blood (which should be less than a week old). Another method is to use a medium containing 5 to 6 per cent agar.

BIOCHEMICAL REACTIONS. *B. proteus* forms acid and usually gas from glucose and does not ferment lactose or mannitol, but the fermentation of saccharose and maltose and the production of indole varies according to the

type of organism, indole production is correlated with maltose fermentation; saccharose fermentation is characteristic of most strains though this property may be lost in laboratory cultures, salicin is fermented by the majority of strains. As noted above *B. proteus* is strongly proteolytic and forms sulphuretted hydrogen in cultures, it reduces nitrates, and ammonia is produced from urea.

Variants. The property of liquefying gelatin may be lost after prolonged artificial cultivation and it may be noted here that some systematists now place in the *Proteus* group the organism which has been generally designated Morgan's bacillus, this organism being non-proteolytic, though under certain conditions it shows the 'spreading' character of growth and has serological relationships to *B. proteus*. Non-motile variants of *B. proteus* 'spread' like those of the may also be distinguished

'X' types. Forms of *B. proteus* have been isolated from the urine in 'X 1' and 'X 2' types, and the serum in often in marked degree. in diagnosis. The agglutination type. *B. proteus* X 19

differs serologically from other strains. It ferments maltose and salicin, and produces indole. A further variety named 'Kingsbury' or 'XK' is agglutinated by the serum from cases of typhus-like illnesses in the Far East and this reaction is also applied for diagnostic purposes. This type does not ferment maltose and salicin, and does not liquefy gelatin. The Weil-Felix reaction will be discussed in the chapter on typhus fever.

Experimental Inoculation. Strains of this organism isolated from septic lesions when injected subcutaneously in laboratory animals produce either a localised inflammatory lesion with pus formation or a spreading inflammatory œdema and septicaemia with fatal result.

Lesions in Human Subject. Although some cases of pure infection by this organism have been described, e.g. pleurisy, meningitis from middle-ear disease, etc., the bacilli generally occur along with other organisms in septic inflammations, such as cystitis and pyelitis, endometritis, peritonitis, etc. *B. proteus* is common also in gun-shot and other contaminated wounds. Cases of gastro-enteritis, including food-poisoning, have been attributed to *B. proteus* by certain observers, and the organism is a frequent concomitant of *B. dysenteriae* infection, occurring often in large numbers in the stools in late stages of the disease.

Organisms which are probably related to *B. proteus* have been described in ozæna, e.g. *Coccobacillus fetidus ozænae* of Perez. They appear to be responsible for the factor and secondary effects in this condition.

BACILLUS FÆCALIS ALKALIGENES (*ALCALIGENES FÆCALIS*)

This organism is a motile, Gram-negative, non-sporing bacillus resembling the coliform bacilli in its morphological and general cultural characters. It is, however, devoid of fermentative properties towards any of the carbohydrate substances used in the testing of the organisms of that group (vide chapter XIII). Indole is not formed. Growing in milk it produces marked alkalinity and on MacConkey's medium the colonies show a yellowish zone round them due to alkali production and colour change of the neutral red. It does not liquefy gelatin. The classical type is actively motile, but reports as to the number and arrangement of its flagella vary. Multiple peritrichous

flagella have been described, but strains are met with possessing a single terminal flagellum like that of certain vibrios. Non-motile strains with otherwise the same characters as *B. fæcalis alkaligenes* may be met with, and it seems probable that organisms classified under this designation represent a number of varieties.

This type of organism may occur occasionally in small numbers in fæces from healthy persons. In certain pathological conditions of the bowel large numbers may be found in the stool, often replacing to some extent the normal coliform flora, e.g. in late cases of bacillary dysentery (q.v.) and in non-dysenteric diarrhœa. Blood infections with *B. fæcalis alkaligenes* have also been described in cases presenting a transient febrile illness.

The taxon

ents some difficulty. American
the *Rhizobiaceæ* (p. 42).

CHAPTER XIII

THE COLIFORM BACILLI AND THE PNEUMOBACILLUS

IN 1885 Escherich described the *Bacillus coli communis*, the classical type of those organisms designated collectively *B coli* or 'coliform' bacilli, which occur normally in the intestine and to which the typhoid bacillus is biologically related. While ordinarily *B coli* is a harmless commensal, it may manifest pathogenic properties under certain conditions. Previously the organism known as *Bacillus typhosus* had been described in 1880-81 by Eberth, who observed its microscopic appearance in the intestinal ulcers and in the spleen in cases of typhoid fever. It was first isolated (from the spleen) in 1884 by Gaffky, and its cultural characters were then investigated. These two organisms belong to a large group (often designated the coli-typhoid group) isolated from the intestinal contents and from various intestinal infections, which bear resemblances to one another, and whose differentiation is in certain cases a matter of some difficulty. Among other members of this group are the paratyphoid bacilli, *B enteritidis* of Gaertner and other organisms associated with a form of 'food poisoning', and the dysentery bacilli.

The general characters of the group are as follows. The organisms, which are microscopically indistinguishable except in regard to motility (and the possession of flagella), this character varying with different members, are non-sporing bacilli whose average size is 2 to 4 μ by 0.5 μ , their flagella when present are distributed all round the bacillus, i.e. peritrichous, they stain with ordinary dyes, and are all Gram-negative. They are aerobes and facultative anaerobes, with a few exceptions, their optimum temperature for growth is 37°C. In solid media they tend to be non-fermentative and do not produce gas, in liquid media they are fermentative and produce gas. This is a property (vide Table I) which is of great importance in their action on sugars and other carbohydrates. A primary classification of the group has been based on the fact that while the typical coliform bacilli produce acid and gas from lactose, the typical pathogenic members have no effect on this sugar, in the ultimate differentiation of these organisms various other biochemical properties and *in vitro* immunity reactions are of essential importance.

In this and succeeding chapters the important organisms to be dealt with will be grouped as follows: (1) those which have been generally designated *Bacillus coli* and occur mainly as commensals in the bowel of mammalian animals, including *Bacillus aerogenes* types, (2) the typhoid and paratyphoid bacilli, associated with enteric fever, (3) the organisms of 'food-poisoning' (acute gastro-enteritis), and (4) the dysentery bacilli. It must be recognised, however, that there is no sharp dividing line as regards biological characters between the enteric fever organisms and those of 'food-poisoning', and in recent classifications these organisms have been placed in one biological group or genus designated *Salmonella*. Their separate consideration here is based on their relationships to different clinical conditions.

BACILLUS COLI OR COLIFORM BACILLI (*ESCHERICHIA*)

The designation *Bacillus coli* is used here as a collective name applicable to a variety of types presenting well-marked common characters though differing in biochemical reactions and in certain other features. *B. coli communis* (Escherich) referred to above represents only one type. It is convenient to describe the group as a whole, indicating the differences among the more important types.

MICROSCOPIC CHARACTERS. These are best seen in young broth or agar cultures. The bacillus is usually 2 to 4 μ long and about 0.4 to 0.5 μ broad; longer forms up to 8 or 10 μ are not infrequent, and short coccobacillary forms are also noted. The size may depend greatly on the medium in which the organisms are growing (Figs. 68, 69). Motility varies with different types and under different growth conditions in the same strain.

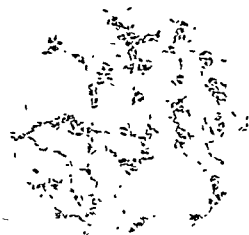


FIG. 68 *Bacillus coli*. Film preparation from a twenty-four hours' culture in glucose broth. Stained with dilute carbol-fuchsin $\times 1000$.

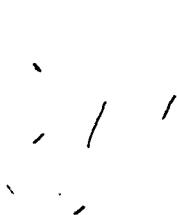


FIG. 69. *Bacillus coli*. Film preparation from a twenty-four hours' culture in dilute peptone water—the same strain as in Fig. 68. Stained with dilute carbol-fuchsin. $\times 1000$.

Some strains are capsulate. The organism may stain somewhat faintly with simple dye solutions but is readily demonstrated with dilute carbol-fuchsin, it is Gram-negative. In older cultures the bacillary protoplasm may be vacuolated and the organism may appear swollen. By appropriate staining the motile varieties can be shown to possess flagella distributed all round the organism, varying in number, but generally less numerous and shorter than those of *B. typhosus*.

CULTURAL CHARACTERS ON ORDINARY MEDIA. Growth occurs on ordinary media under both aerobic and anaerobic conditions; the optimum temperature for most types is 37° C. and the usual temperature range for growth 15° to 44° C., certain types have a slightly lower optimum and do not flourish above 42° C., e.g. strains of *B. aerogenes* (*vide infra*). A stroke inoculation on agar yields after twenty-four hours at 37° C. a somewhat dense, moist, glistening, greyish-white growth along the line of inoculation. On agar plates the surface colonies are relatively large (2 to 3 mm. after twenty-four hours) greyish-white, moist, circular disks; the deep colonies are smaller and lenticular in shape and under a low power of the microscope appear rather dense to transmitted light. Colonies vary in size, thickness, opacity, and structural features among different strains, and in certain cases a mucoid or

viscid character associated with the formation of capsules is observed. In fact, certain types (e.g. *B. aerogenes*) are characterised by their large, slimy, or mucoid colonies (*vide infra*). The colonies may remain circular in outline or become irregular as growth continues. 'Rough' colonies corresponding to the rough variants noted in the typhoid-paratyphoid group may also be observed. In stab culture in gelatin an abundant film-like growth takes place on the surface, and there is a whitish line along the stab without liquefaction of the gelatin (Fig 70). Certain atypical members of the group (e.g. *B. cloacae*) may liquefy gelatin slowly. In broth, typical strains produce a uniform turbidity. On potato, in forty-eight hours there is a distinct film of growth, usually of a brownish-yellow tint, with a moist surface, which rapidly spreads and becomes thicker. The appearance on potato, however, varies much with the different strains and also with the reaction of the potato.

B. coli is facultatively autotrophic, being able to grow even in distilled water by utilising carbon dioxide and ammonia from the atmosphere (Bigger and Nelson).

BIOCHEMICAL CHARACTERS AND CULTURAL REACTIONS IN SPECIAL MEDIA Various media have been used for the appreciation of special characters of the coliform bacilli. These reactions depend upon the capacity of the organism to originate chemical changes on a variety of substances.

Fermentative reactions *B. coli* shows great powers of splitting up carbohydrates with the formation of acids, especially lactic acid, and gases, chiefly carbon dioxide and hydrogen. Certain types, however, classified as *B. coli anaerogenes* (*vide infra*) ferment carbohydrates without gas production. Peptone water or casein digest with an indicator, e.g. litmus, neutral red, or Andrade's indicator, in Durham's tubes, is used, the carbohydrate to be employed being added in the proportion of $\frac{1}{2}$ to 1 per cent. The fermentative capacities of coliform bacilli are very wide and varied. The common types produce acid and gas from glucose, lactose, levulose, galactose, maltose, rhamnose, arabinose, mannitol, xylose, glycerol. Certain varieties ferment saccharose, raffinose, dulcitol, adonitol, inulin, cellobiose, dextrin, and even the benzol derivative inositol (*vide table of sugar reactions*, p. 362). Glycosides may also be fermented e.g. salicin, arbutin. Certain types fail to produce gas when growing in a lactose medium at 44° C (Eijkman's test), though typical strains have this property.

In cultures in gelatin made from fresh meat sometimes bubbles of gas appear from the fermentation of the glucose present in the meat (Fig 70).

The reactions of *B. coli* in some media other than simple sugar solutions likewise depend on sugar fermentation. On MacConkey's bile salt neutral red lactose agar the typical strains of coliform bacilli yield characteristic rose-red colonies due to the fermentation of the lactose and the colour change of the neutral red produced by the acid reaction. Some strains, however, even though they ferment lactose may not yield the typical rose-red colonies within twenty-four hours incubation.

If *B. coli* be grown in milk, e.g. litmus milk, acid is produced from the lactose present, which further curdles the milk. The acid reaction appears to be permanent when growth is allowed to go on for some days.



FIG 70 Stab culture of *Bacillus coli* in gelatin, nine days' growth, the gelatin is split in the lower part owing to the formation of gas. Natural size.

Carbon dioxide : hydrogen ratio. As has been said, the gases produced by *B. coli* in fermenting sugars are chiefly carbon dioxide and hydrogen. Some importance has been attached, first, to the amount of gas formed from a given quantity of glucose in a given time, and, second, to the ratios of the two gases to one another in such a fermentation. According to Harden and others, coliform bacilli may be classified according to their $\text{CO}_2 : \text{H}_2$ ratio. In some cases the ratio is approximately 1 : 1-2—the typical coliform bacilli (*vide infra*); in others 2-3 : 1, as exemplified by *B. aerogenes* (*vide infra*).

For the observation of this the following method can be used. Fermentation tubes, with the closed limb graduated, containing 2 per cent peptone and 1 per cent glucose in tap water, are inoculated and incubated for forty-eight hours at 37° C. The tube is allowed to cool and the total amount of gas noted. The bulb is then filled with 2 per cent sodium hydroxide solution, the opening closed with a rubber stopper and the contents are thoroughly shaken. After the gas has been collected in the closed arm the ratio of the hydrogen left to the original gas volume is read.

Voges-Proskauer reaction. This reaction is not given by the common types of *B. coli*, but as it occurs with certain members of the group it is of importance in differentiating types. It depends on carbohydrate fermentation and is due to the formation of acetyl-methyl-carbinol from glucose. A tube of glucose peptone solution is inoculated and incubated for two days. Then 2 c.c. of a 10 per cent. solution of caustic potash are added and the tube is allowed to stand for several hours or overnight at room temperature. A red fluorescent colour is produced, causing the medium to resemble a weak solution of eosin. The characteristic colour is attributed to a reaction between diacetyl (formed from acetyl-methyl-carbinol in the presence of alkali on oxidation by atmospheric oxygen) and a guanidine constituent of the peptone.

It is stated by some workers that the optimal conditions for the reaction are given by a culture incubated at 30° C for two days.

O'Meara's modification of the test is more delicate than the original method: a minute amount of creatin is added to the culture and then 5 c.c. of 40 per cent sodium hydroxide, and the tube is shaken. A positive reaction is denoted by the appearance within a few minutes of a pink colour, without fluorescence.

Barritt's modification. The medium is glucose phosphate peptone (see Appendix), care must be taken that a suitable peptone is used and that the medium does not itself give a positive reaction. Cultures in 4 c.c. of medium in 6 in. by $\frac{1}{2}$ in. tubes are grown at 37° C for two to three days. Then to 1 c.c. of the culture are added 0.6 c.c. 5 per cent solution of alphanaphthol in absolute alcohol (industrial spirit must not be used) and 0.2 c.c. 40 per cent KOH solution and the mixture is shaken. A positive reaction is shown by a pinkish colour which appears in two to five minutes and deepens to magenta or crimson, reaching its maximum within one hour. Absence of colour at this time or a faint trace of pink, is read as a negative reaction; later a coppery colour develops in the negatives.

Methyl-red reaction. This depends on the limiting hydrogen-ion concentration attained by growth in a standard glucose peptone medium containing a standard amount of dipotassium phosphate, and has been utilised, along with the Voges-Proskauer reaction, in classifying coliform bacilli isolated from water supplies. The common types found in excreta and sewage produce a high concentration, along with a negative Voges-Proskauer reaction. Methyl red added to the culture is used as an indicator of the resulting hydrogen-ion concentration. Organisms producing high and low hydrogen-ion concentrations are described as 'Methyl-red Positive' and 'Methyl-red Negative' respectively. For demonstrating the reaction, the organism is grown for three days in a peptone water medium containing 0.5 per cent.

phosphate,
p to
hour

noted: a red colour indicates a 'positive', a yellow colour a 'negative' reaction. It has been pointed out recently that optimal conditions for the test are provided by a culture which has been incubated at 30° C for five days (Levine).

Production of indole. The typical coliform bacilli produce indole in peptone water. The methods are given in the Appendix, and for the detection of the reaction the use of Ehrlich's rosindole test is most satisfactory. Two peptone water tubes may be inoculated, and if the reaction is not obtained in one after two or three days' growth, the other should be incubated for seven to ten days and then tested. Where a faint reaction is obtained, it is well to corroborate the presence of indole by dissolving the rosindole out with amyl alcohol as described.

Another procedure which some workers regard as more sensitive is to add 1 c.c. of ether to the culture which is then thoroughly shaken so that the indole is extracted and separated by the ether. 0.5 c.c. of Ehrlich's reagent is then added.

Citrate-utilisation. The typical varieties of *B. coli* are unable under certain conditions to use citrate as a source of carbon, but atypical forms, e.g. *B. aerogenes*, possess this property and will grow in a synthetic medium containing sodium citrate. For testing purposes Koser's medium is used: 1.5 grms sodium ammonium hydrogen phosphate, 1 gram potassium dihydrogen phosphate, 0.2 gram magnesium sulphate and 2 grms sodium citrate, made up in a litre of distilled water. A very small inoculum must be used otherwise a fallacious positive result may be obtained. Growth in this medium with visible turbidity within ten days indicates utilisation of the citrate. (For another formula see Appendix.)

Action on neutral red. When *B. coli* is grown in neutral-red lactose broth, a rose-red colour, the effect of the acid upon the dye, is at first seen. Frequently this is suc-

types of *B. coli* though at one time used for the purpose.

Reduction of nitrates. *B. coli* is generally capable of reducing nitrates to nitrites. For this test, a medium is used consisting of 10 grms of peptone in 1 litre of ammonia-free distilled water, and 2 grms of nitrite-free potassium nitrate. The medium is filtered, tubed, and steamed for half an hour on three days. Tubes are inoculated and incubated for forty-eight hours, the formation of nitrites being now tested for by Hoesly's method. The following solutions are required: (a) sulphanic acid, 0.5 gram dissolved in 120 c.c. distilled water and 30 c.c. glacial acetic acid; (b) 0.1 gram *n*-naphthylamine is

study of *B. coli* types

Hæmolysis. According to Dudgeon and others, certain strains of *B. coli* possess distinct hæmolytic properties toward human blood, and these are specially prevalent in acute infections of the urinary system, whereas such hæmolytic strains are uncommon in the faeces. The test for hæmolysis is carried out by growing the organism in peptone solution to which blood has been added.

AGGLUTINATION REACTIONS OF *B. COLI* A study of the agglutination reactions of this group with the sera of animals immunised with various strains, has generally shown an extreme antigenic heterogeneity. Thus, the action of the agglutinins may be restricted to the individual strain used for

immunisation. It has been claimed, however, that hæmolytic strains derived from urinary infections are serologically related. It is of interest that some strains of coliform organisms from feces show H agglutination with antisera to members of the *Salmonella* group.

Pathogenicity of *Bacillus Coli*. Organisms of this group are frequently associated with inflammatory and suppurative lesions in various parts of the body.

Under experimental conditions the virulence of these organisms varies greatly and can be increased by passage from animal to animal. Injection into the serous cavities of rabbits produces a fibrinous inflammation which becomes purulent if the animal lives sufficiently long. If, however, the virulence of the strain be of a high order, death takes place before suppuration is established, and there is a septicæmic condition, the organisms occurring in large numbers in the blood. Intravenous injection of a few drops of a virulent

broth culture usually produces a rapid septicæmia with scattered hæmorrhages in various organs.

Coliform bacilli are found in a great many inflammatory and suppurative conditions in connection with the alimentary tract—for example, in suppuration in the peritoneum, or in the extra-peritoneal tissue with or without perforation of the bowel, in the peritonitis following strangulation of the bowel, in appendicitis and the lesions following it, in suppuration in and around the bile ducts, etc. It may also occur in lesions in other parts of the body—endocarditis, pleurisy, etc., which in some cases are associated with lesions of the intestine, though in others such cannot be found. It is frequently

FIG. 71. Film from urinary sediment showing
B. coli.

Stained with dilute carbol-fuchsin $\times 1,000$.

present in inflammation of the urinary passages, cystitis, pyelitis, abscesses in the kidneys, etc., these lesions being in fact most frequently caused by this group of organisms (Fig. 71). Infection of the pelvis of the kidney may be the sequel to cystitis, i.e. an ascending infection, but cases are common in which the organism has reached the kidney either by the blood stream, or, as has been suggested, by communicating lymphatics from the bowel. Probably small numbers of coliform bacilli may in healthy persons gain access to the lymphatics and even the blood stream, these are rapidly eliminated, but in certain conditions of lowered resistance may become established in the kidney. Obviously the intestinal mucosa presents a high degree of resistance to these organisms whereas other tissues are less resistant. Pyelitis due to coliform bacilli is frequent in female infants and presents characteristic clinical features. Coliform bacilli are also found in suppurating wounds, but usually in association with other pyogenic bacteria. An acute febrile illness has followed inhalation of dust from low-grade cotton heavily infected with a bacillus, this is apparently due to toxic products

cultivated from various lesions are more virulent than the ordinary intestinal strains, their virulence having been heightened by growth in the tissues. Sanarelli found that strains from typhoid stools were more virulent for laboratory animals than those isolated

from healthy persons. Coliform organisms are probably active secondary invaders when the mucous membrane is damaged by some other agent, as in enteric fever, dysentery, etc

While coliform bacilli are normally harmless in the intestine, it seems

Isolation of *B. Coli*. In the case of abscesses or infection of the kidney or bladder, etc., the isolation of the organism is easily accomplished by inoculating plates of MacConkey's bile-salt neutral-red lactose agar with the pus or centrifuged deposit from urine. The colonies are usually characterised by their rose-pink colour. Where there is a mixed infection with such organisms as streptococci, for diagnostic purposes cultures should be made also on other media, e.g. blood agar, to obtain a satisfactory growth of these as well as *B. coli* since the bile-salt of MacConkey's medium may inhibit the associated pyogenic organisms.

The Recognition of Typical Coliform Bacilli and the Classification of these Organisms. The work on *B. coli*, especially in relation to its occurrence in water, has revealed the existence of a very large number of varieties of the organism. These differ from one another in certain biological characters, e.g. motility, fermentation reactions with sugars, indole formation, etc (*vide infra*). Some difference of opinion has existed as to what characters are to be looked upon as typical characters, i.e. characters shared by the greatest number of varieties present in faeces and therefore, when present, significant of excretal contamination.

Two of the original standards may be alluded to. First, that of an English Committee who reported in 1904 on the standardisation of methods for the bacteriological examination of water. According to them, *B. coli* is a small, motile, non-sporing bacillus, capable of growing at 37° C, Gram-negative, never liquefying gelatin, producing clot and permanent acidity of milk within seven days at 37° C, fermenting glucose and lactose, with, in both, acid and gas formation—subsidiary characters being the formation of indole, the formation of a thick yellowish-brown growth on potato, production of fluorescence in neutral-red media, and reduction of nitrates. A similar American Committee looked upon the typical organism as a non-sporing bacillus, motile, fermenting glucose-broth, with the formation, in the closed limb of the fermentation tube, of about 50 per cent of gas, of which about one-third is carbon dioxide, causing acid and clot in milk in forty-eight hours, not liquefying gelatin, producing indole, and reducing nitrates.

From investigation not only of strains isolated from pathological conditions, but in connection with the bacteriology of water, milk, and faeces, it had been found that a very large number of Gram-negative bacilli exist which have in common the capacity of fermenting glucose and lactose, but which also present individual differences. The work of MacConkey first opened the way to the practical classification of these organisms. It was shown that certain of the tests originally applied gave little significant information, e.g. observation of fluorescence in neutral-red lactose media (on account of the inconstancy of the occurrence of this change), the reduction of nitrates (this being a common property of nearly all the members of the

to the following criteria: motility, fermentation of dulcitol, saccharose, adonitol, inulin, inositol, the formation of indole, the Voges-Proskauer reaction.

and liquefaction of gelatin. This classification of MacConkey elicited the fact that certain biological types are specially prevalent in the faeces of man and animals; e.g. the type designated by him No. 71, which corresponds to the organism often spoken of in bacteriological literature as *B. coli communior* (*Escherichia communior*), and also *B. coli communis*, Escherich (*Escherichia coli*). The only difference between the two is that No. 71 forms acid and gas from saccharose. (*vide* table of characters, p. 362.)

Typical ('faecal') coliform bacilli. Studies of this group have now shown that the most prevalent and characteristic coliform types found in faeces present the following common positive and negative characters: fermentation of lactose with gas production, production of indole, absence of fermentation of inositol, absence of gelatin liquefaction, a $\text{CO}_2 : \text{H}_2$ ratio of 1:1-2, a negative Voges-Proskauer reaction, a positive methyl-red reaction, non-utilisation of citrate in Koser's medium, and ability to produce gas from lactose at 44°C . (Eijkman reaction). The recognition of these characters

have in common the property of fermenting inositol, and thus affords an important classificatory criterion. Such organisms are exemplified by *B. (lactis) aerogenes* (*vide* Table, p. 362). This type was originally described by Escherich in his work on the intestinal flora of children; he pointed out that

colonies and fermentation of adonitol. In addition, the Voges-Proskauer reaction is frequently positive, the $\text{CO}_2 : \text{H}_2$ ratio is approximately 2:1, the methyl-red reaction is usually negative and citrate-utilisation is a characteristic feature. These types have a somewhat lower optimum temperature than the typical coliform bacilli and do not produce gas from lactose at 44°C . (negative Eijkman reaction). It has been pointed out, however, that in climates with a high mean temperature strains isolated from water may give a positive Eijkman reaction (Boizot). The separate designation, *B. aerogenes* (*Aerobacter*) is often applied to all such organisms. To this subgroup the capsule Gram-negative bacilli found in the respiratory tract are closely related, e.g. the pneumobacillus.

The question arises as to whether gelatin-liquefying, Gram-negative bacilli which correspond in other characters to *B. coli* are to be included in the coliform group. Among the lactose-fermenters classified by MacConkey certain gelatin liquefiers, otherwise resembling *B. aerogenes*, were represented, e.g. *B. cloacae*, etc., and various authorities have accepted this reaction as one of the possible characters of the group. Such organisms may be regarded, however, as quite atypical.

Another category of gas production is absent

anaerogenes (*vide* Table, from the group of dysentery bacilli presents some difficulty.

Significance of typical and atypical coliform bacilli. Thus, among the coliform bacilli there are on the one hand the typical forms, as defined above, which predominate in the faeces of man and animals, and on the other, atypical organisms, exemplified by *B. aerogenes*, which are relatively scanty in the intestine of the healthy animal. Between these extremes, however, there are many intermediate atypical forms, which are also relatively infrequent in faeces. From bacteriological studies of water

supplies it has been shown that while the typical forms have only a limited power of survival in ordinary environments outside the body, the atypical organisms are often more 'resistant' and the latter are therefore found fre-

Thus, 'faecal' and 'non-faecal' respectively, but such nomenclature may be somewhat misleading. It must be emphasised that practically all varieties have been found in the intestine; and atypical forms even when not detectable in faeces by ordinary methods can be demonstrated readily by selective enrichment. Further, in abnormal conditions of the intestine the atypical forms may predominate (see Krishnan and Chawla). The separation of typical from atypical coliform bacilli is nevertheless of the greatest value when *B. coli* is utilised as an indicator of recent faecal contamination. In this case the presence of typical organisms is significant, the occurrence of atypical forms is of less significance.

While the characterisation of the typical coliform bacilli given above is generally applicable, the importance attached to particular properties has varied. Special stress has been laid by some writers on citrate-utilisation as a feature of the 'non-faecal' types. This property is certainly uncommon among strains isolated from faeces, while it is an almost constant feature of the *B. aerogenes* subgroup. On the other hand citrate-utilising organisms, otherwise typical (the so-called 'intermediate' types) may sometimes occur in the intestine; moreover typical coliform bacilli may acquire the property of utilising citrate. Carpenter and Fulton, from a study of 'intermediate'

of coliform bacilli of bovine origin that all inositol-fermenting strains utilise citrate, while among the non-inositol fermenters there is a close negative correlation between citrate utilisation and the indole reaction. Indole formation has been generally accepted as an important criterion in the recognition of typical *B. coli*. In characterising excretal coliform bacilli from water Bardsley, however, has considered that a negative indole reaction is insufficient by itself to exclude an organism from this category. Kulp kept strains of *B. coli* in sterilised soil and found indole production variable, sometimes positive, sometimes negative. Another feature of the typical *B. coli* which has been referred to by some workers is the absence of fermentation of cellobiose, a property possessed by various atypical organisms, but it is doubtful whether this reaction differentiates between 'faecal' and other strains. Recently Wilson and his co-workers have classified strains into subgroups on the basis of the methyl-red, Voges-Proskauer, citrate, indole, Eijkman, and gelatin-liquefaction tests. Thus strains of lactose-fermenting coliform bacilli which are methyl-red positive, Voges-Proskauer negative, non-citrate-utilising, and indole-positive, constitute the typical or faecal coliform bacilli. Strains similar to the above except that they are indole-negative are regarded as doubtful. Strains which are methyl-red negative, Voges-Proskauer negative, and citrate-utilising are regarded as *B. aerogenes*. Strains which are methyl-red negative, Voges-Proskauer negative, and citrate-utilising are regarded as *B. freundii* in the American nomenclature. The two latter categories are regarded as non-faecal. For further details their monograph should be consulted. While such classifications are of practical value in the examination of water supplies with reference to recent pollution with sewage, it must be borne in mind that from a biological point of view they may be to a considerable extent arbitrary.

Paracolon bacilli. This designation has been given to coliform bacilli which differ from the typical organisms mainly in respect of the absence of lactose fermentation. Some of these organisms, however, may show late fermentation of this sugar and the organism designated *B. coli mutabilis* (*vide infra*) might be placed in this group. Such types are infrequently found in normal faeces, and hence their presence, often in large numbers, in the stools in pathological conditions, e.g. enteric fever, dysentery, etc., has attracted special attention, particularly in tropical countries. This has suggested that they may have a secondary pathogenic rôle. They have also been observed in large number in simple diarrhoeal conditions, in urinary infections and even blood infections, and the possibility of their having primary, though weak, pathogenic properties has been considered likely by various observers. They have been regarded as biologically intermediate between the true coliform bacilli and the specific intestinal pathogens. In diagnostic work they come under review in the first place because their colonies on a lactose medium with acid indicator, such as MacConkey's, are similar to those of the typhoid-paratyphoid and dysentery bacilli, and moreover some strains exhibit biochemical reactions similar to those of the paratyphoid bacilli (*vide Table, p. 362*), e.g. fermenting glucose and mannitol with gas production and not fermenting lactose and saccharose, certain of them also ferment dulcitol. They can often be differentiated, however, from the paratyphoid organisms by the fermentation of saccharose, indole production, and by late lactose fermentation. Some strains are motile, others non-motile. As regards the indole, methyl-red, Voges-Proskauer and citrate-utilisation reactions, some correspond to the typical *B. coli*, others are like *B. aerogenes* and, but for the absence of lactose fermentation, many would be regarded as true coliform organisms. The fundamental question arises, therefore, as to the significance to be attached to lactose fermentation as compared with other biochemical reactions, all these so-called paracolon bacilli might justifiably be classed as atypical coliform bacilli. Serological tests usually separate them clearly from the specific pathogenic intestinal bacilli. It is of interest, however, that known *Salmonella* antigenic constituents may be found among these organisms (see Edwards *et al.*, Stuart *et al.*); also they do not show the same degree of serological heterogeneity as other coliform bacilli. Some paracolon strains contain the typhoid Vi antigen (Marmion).

Variation and mutation in the *B. coli* group has been referred to in Chapter I. Variation in fermentative properties is well illustrated by the type named *B. coli mutabilis* in which colonies develop papillae differing from the parent strain in the power of fermenting lactose and from these papillae a stable variant is derived

be lost. In certain

different colonies when these are cultivated separately. Variability in the characters of practically all characters of these organisms has been recorded and is certainly an outstanding feature of the group. While some strains exhibit marked variability, others may remain stable for long periods in culture. It is of course possible that variation is most active in their natural habitat and that laboratory cultures become stabilised. According to Parr and Caldwell when coliform bacilli are subjected to a sudden and marked environmental change, variants are formed giving rise to apparently different biochemical types. D'Herelle has recorded such variation under the influence of bacteriophage and suggests that *B. coli* phages present in water may even bring about a mutation of *B. coli* to *B. aerogenes*. Variation in colony characters is also frequently noted, and, as in other groups, there may be dissociation with partial or complete loss of the original antigenic characters

PNEUMOBACILLUS OF FRIEDLÄNDER (KLEBSIELLA PNEUMONIÆ)

This organism is of historic interest, as it was the first organism cultivated from pneumonia, and it was confused with the pneumococcus. It does not occur alone in more than about 1 per cent of cases of pneumonia, in the pneumonia caused by it the consolidated lung has often a somewhat slimy or mucoid character. It is biologically related to the coliform bacilli.

MICROSCOPIC CHARACTERS In the sputum it may appear as a very short diplobacillus (1 to 2 μ by 0.5 to 0.8 μ) possessing a well-developed capsule, but it is also frequently seen in the form of long rods. It is Gram-negative, non-motile, and non-sporing (Fig 72). Capsules can be demonstrated in cultures by relief staining (Fig 73).

CULTURAL CHARACTERS It is an aerobe and facultative anaerobe, and can be easily isolated on agar plates, on which it forms large whitish moist

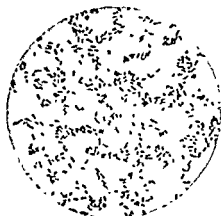


FIG 72. Friedländer's pneumobacillus, from a young culture on agar, showing rod-shaped forms. Stained with carbol-thionin $\times 1,000$

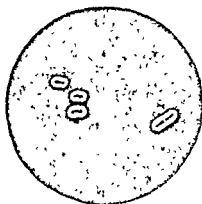


FIG 73. Friedländer's pneumobacillus film from a culture. Cochin capsule method $\times 1,000$

viscid colonies (cf *B. aerogenes*). Non-mucoid variants may be derived from the original mucoid organism. In gelatin stab, at the site of the puncture the growth is sometimes heaped up above the level of the medium, and along the wire track there is a white granular appearance. The gelatin is not liquefied. The organism grows well on all ordinary media.

BIOCHEMICAL REACTIONS There is some variation among different strains as regards their fermentative reactions. Certain strains conform in their biochemical characters to the inositol-fermenting subgroup of the coliform bacilli (vide Table, p. 362) and ferment glucose, lactose, saccharose, adonitol, and inositol, usually with gas production. Some strains, however, fail to ferment lactose and some do not produce gas from fermented carbohydrates. Formation of indole from peptone is variable. The same applies to the Voges-Proskauer and methyl-red reactions. It might be said, however, that the majority of strains yield a negative indole reaction, a positive methyl-red reaction, and negative Voges-Proskauer reaction. Some strains utilise citrate and most grow on bile-salt media, e.g. MacConkey's. It is difficult to draw any sharp distinction between these capsulate Gram-negative bacilli from the respiratory tract and similar forms occurring in the intestine, e.g. *B. aerogenes*.

By *serological reactions* with antisera, it has been shown that strains of this organism from cases of pneumonia can be classified into three types (A, B, and C) and a fourth heterogeneous group. As in the pneumococcus, specificity depends on a carbohydrate substance associated with the capsule, and it is of special interest that Type B is serologically related to pneumococcus Type II (Julianelle)

PATHOGENICITY. When injected into mice and guinea-pigs it causes a septicæmia and can be seen in the heart blood to possess a capsule. It is less pathogenic to rabbits and dogs, but when injected into the trachea in these animals it originates a pneumonia. Type C is apparently of less virulence than Types A and B. As stated above, it is the only organism present in a small number of cases of human pneumonia, and it has also been isolated from conditions of empyema, meningitis, suppuration of nasal sinuses, conjunctivitis, bronchitis, and rarely in septicæmic conditions. It is a not infrequent inhabitant of the mouth and nose of healthy individuals.

THE BACILLUS OF RHINOSCLEROMA (*KLEBSIELLA RHINOSCLEROMATIS*)

This organism is considered here as it is biologically related to the pneumobacillus. Rhinoscleroma belongs to the group of infective granulomata. It is characterised by the occurrence of chronic nodular thickenings in the skin or mucous membrane of the nose, or in the mucous membrane of the pharynx, larynx, or upper part of the trachea. The nodules are of considerable size, sometimes as large as a pea; in the earlier stages they are comparatively smooth on the surface, but later they become shrunken and the centre is often retracted. The disease is scarcely ever met with in this country, but has been reported on the Continent, especially in Austria and Poland. In the granulation tissue of the nodules there are to be found numerous round and rather large cells, which have peculiar characters and are often known as the cells of Mikulicz. Their protoplasm contains a collection of somewhat gelatinous material which may fill the cell and push the nucleus to the side. Within these cells there is present a characteristic bacillus, occurring in little clumps or masses, chiefly in the gelatinous material. A few bacilli also lie free in the lymphatic spaces around. This organism, known as the bacillus of rhinoscleroma, was first observed by Frisch. The bacilli have the form of short oval rods, which, when lying separately, can be seen to possess a distinct capsule; in their microscopic characters they correspond closely with Friedländer's pneumobacillus. They are Gram negative, and as a rule they are the only organisms demonstrable.

From the affected tissues this bacillus can be easily cultivated by the ordinary methods. In the characters of its growth on the various culture media it presents a close similarity to the pneumobacillus, as it does also in its fermentative actions. It is non-gas producing and does not ferment lactose; the indole and Voges-Proskauer reactions are negative; it has been stated that the bacillus of rhinoscleroma is inhibited by bile-salt.

The serum of patients suffering from the disease gives fixation of complement when tested with an emulsion of the bacillus, but varying results have been obtained as regards the validity of the test in the differentiation of the bacillus from allied organisms.

The evidence that the organisms described are the cause of this disease consists essentially in their constant presence and their special relation to the affected tissues, as already described. Experimental inoculation has thrown little light on the subject, though one observer has described the production of nodules in the conjunctivæ of guinea-pigs.

Recent investigators consider the bacillus of rhinoscleroma to be identical with the pneumobacillus, and its presence in the affected tissues to represent merely a secondary invasion.

BACILLUS OZÆNÆ (*KLEBSIELLA OZÆNÆ*)

A bacillus possessing closely similar characters to those of the pneumobacillus has been found to be very frequently present in ozæna. It is non-gas-producing in fermentation tests. Its relationship to ozæna is probably that of a secondary organism. The bacillus of Perez which has also been described in ozæna is referred to on p. 309.

From what has been stated regarding the rhinoscleroma bacillus and *B. ozænæ* it will be seen that a number of closely allied organisms have been found in the nasal cavity in healthy or diseased conditions.

CHAPTER XIV

THE ENTERIC GROUP (THE TYPHOID BACILLUS AND THE PARATYPHOID BACILLI) AND THE SALMONELLA GROUP OF FOOD-POISONING ORGANISMS

REFERENCE has been made in the introduction to Chapter XIII to the dis-

but distinguishable from, the typhoid bacillus. It soon became recognised

Infections due to them have sometimes been termed 'paratyphoid fever', but clinically they may be indistinguishable from true typhoid though they frequently take the form of a milder and less prolonged illness. The pathological changes, though often less pronounced than in typhoid, are also similar and it has become customary to include true typhoid and the paratyphoid infections under the single clinical and epidemiological designation 'enteric fever'. In this section the characters of the typhoid bacillus will first be described, and then the paratyphoid organisms, with particular reference to their differences from the former and from one another. The relationship of the typhoid-paratyphoid bacilli to enteric fever will be discussed along with certain epidemiological features of the disease and the subject of immunity to it.

BACILLUS TYPHOSUS

(*EBERTHELLA TYPHOSA* OR *SALMONELLA TYPHI*)

MICROSCOPIC CHARACTERS. As observed in pure culture and in the tissues, the individual organisms are straight, cylindrical or rod-shaped structures, with rounded ends, measuring on an average 2 to 4 μ long and 0.5 μ in thickness. Frequently in culture filamentous forms are noted, even 10 μ or more in length. In culture the bacilli occur singly and in pairs end to end (Fig. 74). In tissues, e.g. in a Peyer's patch, spleen, liver, or mesenteric gland, they are found in relatively large clumps (*vide* Fig. 75). For their demonstration in tissue sections, carbol-thionin may be used, or prolonged staining with Löffler's methylene blue (half an hour), the sections being dehydrated and cleared with aniline-xylol. The typhoid bacillus is Gram-negative and non-sporing.

In hanging-drop preparations the bacilli are found to be actively motile. The smaller forms have a darting or rolling motion, passing quickly across the field, while some show rapid rotatory motion. The filamentous forms have an undulating or serpentine motion, and move more slowly. Hanging-drop preparations should be made from agar or broth cultures, preferably within six to twelve hours after incubating at 37° C. and not more than twenty-four hours old. In older cultures the movements are less active.

On being stained by appropriate methods the bacilli are seen to possess multiple long peritrichous flagella (Fig. 76). According to Pijper the typhoid

bacillus as observed by solar dark-ground illumination exhibits only two flagella, one on each side, which project from the middle of the organism; he describes them as spiral structures which are intertwined so as to form a long tail-like filament by which the bacillus is propelled through a fluid medium. It seems likely that the flagella so described are composite structures, their constituents being observed as peritrichous threads in preparations stained

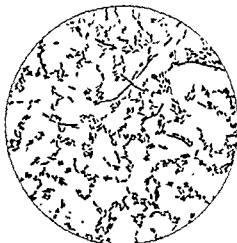


FIG. 74. Typhoid bacilli, from a young culture on agar, showing some filamentous forms. Stained with dilute carbol-fuchsin. $\times 1,000$.

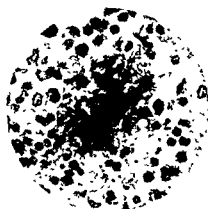


FIG. 75. A large clump of typhoid bacilli in a spleen. The individual bacilli are only seen at the periphery of the mass. Paraffin section stained with carbol-thionin $\times 500$.



FIG. 76. Typhoid bacilli, from a young culture on agar, showing flagella. Stained by Kirkpatrick's method $\times 1,000$.

for flagella by the usual methods, and that in the movement of the organism the multiple flagella become twisted together to form the tail-like structure described by Pijper.

In a more recent publication Pijper suggests that bacterial flagella are not locomotory organs, but processes derived by movement from a slime-layer covering the cell, and that the motive force consists of wave-like contractions of the cytoplasm.

CULTURAL CHARACTERS. To grow the organism artificially from cases *post mortem* it is best to isolate it from the spleen, or from the bile in the gall-bladder, as it exists in these situations in greater numbers than in the other organs, and may be the sole organism present even some time after death. The obtaining of pure cultures from cases during life will be dealt with in

detail later. The organism is an aerobe and facultative anaerobe: its optimum temperature is about 37° C and the temperature range for growth 15° to 41° C. It can be cultivated on all the ordinary media.

Generally speaking, growths of *B. typhosus* appear less dense and abundant than those of the *B. coli*. In stroke cultures on agar there is a greyish-white film of growth, with fairly regular margins, but without any characteristic features (Fig. 77). This film is moist, loosely attached to the surface, and can be easily scraped off and emulsified. Colonies after twenty-four hours' incubation are semi-transparent, circular disks, about 2 mm. in diameter and normally exhibit a smooth surface and well-defined border, but if growth is allowed to continue for a few days they enlarge and may develop an irregular margin, a raised centre, and often radial ridges, this type



FIG. 77. Stroke culture of *B. typhosus* on agar, twenty-four hours' growth. Natural size.

of colony has been likened to a vine leaf. A certain amount of variation, as regards details of structure, may be observed among different strains. Stab cultures in gelatin yield the following appearance: on the surface of the medium growth spreads outwards from the puncture as a thin leaf-like film, with irregularly wavy margin; it is semi-transparent and of bluish-white colour, ultimately this surface growth may reach the wall of the tube; not infrequently, however, the surface growth is less marked, along the stab there is an opaque whitish line of growth, of finely nodose appearance, there is no liquefaction of the medium (Fig. 78). On gelatin, surface colonies are more transparent than those on agar but present similar features. In broth the typhoid bacillus normally produces a uniform milky or turbidity. On potato, for several days (at incubator temperature) there is apparently no growth, but if looked at obliquely the surface appears wet, and when it is scraped with the wire loop, a glistening track



FIG. 78. Stab culture of *B. typhosus* in gelatin, five days' growth. Natural size.

is left. A film preparation shows numerous bacilli. Later, however, a slight pellicle with a dull, somewhat velvety surface may appear. These appearances are only seen when fresh potato has been used. On MacConkey's bile-salt neutral-red lactose agar and desoxycholate-citrate agar (also containing lactose and neutral-red) the colonies resemble those on ordinary agar. They remain colourless or 'pale' as compared with those of *B. coli*, owing to the absence of lactose fermentation.

Colony variants. The typhoid bacillus, like other members of the colityphoid group, when cultivated artificially tends to undergo the variation involving the transformation of colonies from the normal smooth (S) type to the rough (R) form (Fig. 79). Growths from the R colonies are spontaneously agglutinable in normal saline (though forming permanent suspensions in weaker saline) and form in broth a granular sediment with clear fluid and sometimes a surface scum, in these respects differing from the S growths, which give a uniform turbidity. The two types differ also in their antigenic structure and their agglutination reactions. These properties tend to be maintained in subcultures, but the S type may yield R variant colonies on continuous cultivation. A variant has also been

described whose colonies are unusually small, not exceeding 0.3 mm after forty-eight hours; in other respects, however, this variant has the characters of standard strains of *B. typhosus*.

BIOCHEMICAL CHARACTERS. The typhoid bacillus can be differentiated from *B. coli* and the other members of the coli-typhoid group by biochemical reactions (*vide* Table, p. 362). It produces *acid without gas* in glucose, levulose, galactose, dextrin, maltose, and mannitol, but originates no change in lactose, saccharose, or rhamnose. In xylose the reaction is variable. In arabinose and dulcitol late acid formation has been observed. On media containing these carbohydrates colonies may develop papillæ which differ from the parent strain in fermenting the particular substances; these are analogous to the lactose-fermenting papillæ of *B. coli mutabilis* (*q.v.*) There is no curdling of milk, although at first slight acid production occurs, in a variable time the acid change may be succeeded by a neutral reaction. The typhoid bacillus does not apparently produce indole in peptone solution; but it has been pointed out by Burrows that indole is actually formed though



FIG. 79. A, smooth, and B, rough type of colony of *B. typhosus*. $\times 20$

not in sufficient amount to give a reaction with Ehrlich's rosindole reagent. *B. typhosus* usually forms hydrogen sulphide in culture medium. Certain salts of organic acids are utilised by this organism.

VIABILITY The typhoid bacillus is similar to most non-sporing bacteria in its powers of resistance to environmental influences. It is killed by exposure for ten minutes at 56° C. and almost instantaneously at 100° C. It has generally been found to be sensitive to natural dessication, but according to Dold and Ketterer when drops of fluid stools from enteric cases are allowed to dry on filter paper, *B. typhosus* may remain viable for an average period of eighty days and *B. paratyphosus B* for 174 days. When kept in water it has been stated that viability of the typhoid bacillus does not persist for more than three weeks, but it must be recognised that duration of survival in water depends on various factors, *e.g.* temperature, presence of organic matter, other bacteria, etc. Beard has pointed out that in sewage-polluted sea and fresh water viable typhoid bacilli may be detected after four weeks. Cultures remain viable for several months.

SEROLOGICAL CHARACTERS *Bacillus typhosus* possesses both somatic (O) and flagellar (H) antigens, and immunisation with the organism leads to the formation of agglutinins which react specifically with these antigens. The O antigenic constituents of the typhoid bacillus are common to certain other members of the *Salmonella* group. The H antigen also is common

to certain *Salmonella* types, but these possess different O components. The identity of the typhoid bacillus can therefore be conclusively established by means of agglutination and agglutinin-absorption reactions. It may be said that if the serum of an animal immunised with a known strain of *B. typhosus* agglutinates an organism with the cultural and biochemical characters of this species, to the same titre as the known strain, the organism in question is almost certainly the typhoid bacillus. Further reference will be made later to the serological differentiation of the typhoid bacillus from allied types.

Freshly isolated strains of *B. typhosus* which are more virulent to mice than stock cultures, possess an additional somatic antigen which has been designated 'Vi' in virtue of its association with virulence (Felix). The presence of this antigen seems to render the organism relatively inagglutinable by an O antiserum, but such inagglutinability disappears on subculture in parallel with the loss of the Vi antigen. This antigen is destroyed by ten minutes' exposure at 100° C., it is also partially destroyed by phenol. Strains produce it best when growing in a soft ascitic-fluid agar. A pure Vi antiserum can be obtained by immunisation with a living culture known to contain the Vi antigen and by then absorbing from the serum H and O agglutinins with strains containing only the corresponding antigens, such a

and that the therapeutic action of a typhoid antiserum is likewise dependent on the presence of the Vi antibody (*vide infra*).

It is of interest that some strains of paracolon bacilli have been found to contain the typhoid Vi antigen (Marmion).

BACILLUS PARATYPHOSUS A (*SALMONELLA PARATYPHI*) and BACILLUS PARATYPHOSUS B (*SALMONELLA SCHOTTMÜLLERI*)

These are the main types of paratyphoid organisms, the latter being the commoner in Europe, the former being more prevalent in the East. In recent times in this country *B. paratyphosus B* has been a more frequent cause of enteric fever than the typhoid bacillus. These paratyphoid bacilli resemble *B. typhosus* closely in their biological characters, but differ from it and from one another in biochemical reactions and antigenic structure. For descriptive purposes only the points of difference need be dealt with. They produce acid and gas in glucose, levulose, galactose, mannitol, maltose, dulcitol, sorbitol, and arabinose; they do not ferment lactose, saccharose, raffinose, salicin, or inulin. Of these reactions, that with lactose differentiates them from the common types of *B. coli*, and the production of gas distinguishes them from *B. typhosus* and *B. dysenteriae*. They do not produce indole, though indole-forming variants have been described by certain observers.

The fermentative properties of *B. paratyphosus A* and *B* are in general similar, but *A* is the less active—gas formation being often scanty and late in appearance. This is particularly noticeable in regard to dulcitol. In skim milk *A* produces little or no change, while in the case of *B* there is rapid alkali production. The *B* type ferments xylose and usually ferments inositol and produces sulphuretted hydrogen (as observed in lead acetate agar) while *A* lacks these properties (*vide Table*, p. 362). Strains of *B* which have been

isolated from cases of gastro-enteritis are stated to be able to utilise *d*-tartrate (*vide* p. 345), whereas enteric strains lack this property. Some strains of the *B* type utilise sodium citrate; *A* does not.

Colonies of *B. paratyphosus B* frequently develop a striking character under certain conditions and in this respect differ from colonies of *B. paratyphosus A*, and *B. typhosus*. This consists in the formation of an opaque raised margin developing when plates that have been incubated at 37° C. are subsequently allowed to stand at room temperature for twenty-four to forty-eight hours. This character, 'schleimwallbildung' (Müller), may assist in the recognition of the colonies and the isolation of the organism from mixed growths (Fig. 80). As has been described in the case of the typhoid bacillus the S→R transformation of colonies is observed in cultures

These paratyphoid bacilli differ in their antigenic structure from one another and also from *B. typhosus*, and in the ultimate differentiation of the two types their reactions with specific agglutinating sera are of essential importance. They show, however, certain serological relationships with other members of the *Salmonella* group, e.g. the organisms concerned in 'food-poisoning,' and this question is considered later.



FIG. 80. Colonies of *B. paratyphosus B* after twenty-four hours' growth at 37° C. and forty-eight hours further at room temperature—showing the characteristic raised margin $\times 1\frac{1}{2}$.

Bacillus paratyphosus C and other Organisms of Enteric Fever. Cases of enteric fever have also been reported in various parts of the world as being due to a type of paratyphoid bacillus which is biologically different from the others, and this organism has been designated *B. paratyphosus C*. It is closely related to *B. suispestifer* (though different in certain respects) and will be dealt with later along with that organism. Certain further types referable to the *Salmonella* group, though differing in antigenic structure from the classical paratyphoid bacilli, have

occasionally been recorded in cases of enteric fever, e.g. *B. suispestifer* (Kunzendorf type), Bareilly, and Eastbourne types. Another *Salmonella* type, designated the 'Sendai' type, has been observed in Japan. It is similar to the other paratyphoid bacilli in general characters, but differs in its antigenic structure. These organisms will be referred to along with the food-poisoning organisms.

RELATIONS OF TYPHOID-PARATYPHOID BACILLI TO ENTERIC FEVER

Pathogenic Effects produced in Animals. Most of the experimental work on this subject has been done with *B. typhosus*. There is no disease of animals which is identical with typhoid, nor is there any evidence of the occurrence of the typhoid bacillus under ordinary pathological conditions in the bodies of animals, and the same is true for *B. paratyphosus A* and *B.* Attempts to communicate the disease to animals by feeding them on typhoid dejecta have been unsuccessful, and though pathogenic effects have been produced by introducing pure cultures of *B. typhosus* in food, the disease has usually borne no resemblance to human typhoid. The results of subcutaneous or intraperitoneal injection are no more satisfactory

The type of disease is very different from what occurs naturally in man, and is often merely an acute toxæmia due to the endotoxin present in the organisms. In such injection experiments the results vary considerably—no doubt due to the fact that different strains of the bacillus vary much in virulence, ordinary laboratory cultures being often almost non-pathogenic. Reference has been made above to the virulence for mice of recently isolated strains containing the Vi antigen. Certain workers using highly virulent strains have been able by intraperitoneal injection to produce in animals effects on the Peyer's patches, mesenteric glands, and spleen similar to those in the disease in the human subject. It has also been found by experiments in rabbits in which recently isolated cultures of *B. paratyphi* that a pathological condition could be reproduced, the mesenteric glands, and the spleen. Experiments with *B. paratyphosus* *B. typhosus* have indicated that this organism is of higher virulence to mice than *B. typhosus*, when introduced by feeding it may produce a non-fatal infection. Metchnikoff and Besredka produced in monkeys a mild condition similar to human typhoid fever by feeding the animals with fæces containing typhoid bacilli or large doses of cultures of the typhoid bacillus. It is especially noted.

marrow after intravenous injection and persist there after they have disappeared from other parts of the body.

The Toxic Products of the Typhoid and Paratyphoid Bacilli. There exist in the bodies of these organisms toxic substances which in artificial cultures do not pass out to any great degree into the surrounding medium, unlike the exotoxins they have no specific effects. The bacterial bodies killed by chloroform vapour are very toxic—more so than filtered cultures—and there is evidence of the release of poisons from the organisms when these undergo lysis in the animal body. Macfadyen, by grinding up typhoid bacilli frozen solid by liquid air, produced a fluid whose toxic effect he attributed to the presence of the intracellular poisons.

Light has been thrown on the chemical nature of the toxic products of the group of organisms to which the typhoid-paratyphoid bacilli belong, by studies of chemical fractions isolated from *B. aertrycke* and *B. enteritidis* (*vide infra*). Such work has indicated that the toxic principle of these organisms is a complex polysaccharide combined with phosphatide, this substance being also antigenic, in fact it constitutes the somatic antigen of the organism (Boivin *et al.*, Raistrick and Topley). In rabbits the toxic antigen produces a marked hyperglycæmia, succeeded often by hypoglycæmia. It is of interest that the polysaccharide can be separated from the phosphatide with which it is combined, and both fractions individually are practically non-toxic. These findings are of special significance in defining endotoxins of known chemical constitution, but of course other still undefined toxic substances may be present in the bacterial cells.

The Course of the Infection and Pathology of Enteric Fever. Infection takes place by ingestion of the organisms and their passage into the small intestine where they invade the tissues and lymphatics and so reach the general circulation, producing the bacteræmia which is a characteristic feature of the early stage of the disease, i.e. during the first seven to ten days. At this period, especially in the first half of it, the organisms can be readily isolated by blood culture. This is also the case in relapses. From the blood they

localise in the liver, spleen, kidneys, bone marrow, and other tissues. As immunity reactions develop the bacteriæmia declines. From the liver capillaries and bile canaliculi the bacilli reach the gall-bladder and multiply in the bile, and at this stage large numbers are discharged into the intestine and appear in the fæces. This is most pronounced in the third week of the illness. As will be shown later, the organism may persist in the gall-bladder and sometimes in other tissues after apparent recovery. Late in the disease the organisms tend to appear in the urine.

The inflammation and ulceration in the *Peyer's patches and solitary follicles of the small intestine* are important pathological features. In the early stage there is an acute inflammatory condition, and infiltration with mononuclear cells, sometimes attended with small hæmorrhages. At this period the bacilli are most numerous in the patches, groups being easily found between the cells. The subsequent necrosis may be due to the action of toxic bacterial products, the bacilli, however, gradually disappear, though they may still be found in the deeper tissues and at the spreading margin of the necrosed area. They also occur in the lymphatic spaces of the muscular coat. The number of the ulcers arising in the course of a case bears no relation to its severity. Small ulcers may occur in the lymphoid follicles of the large intestine. In *B. paratyphosus* *B. infections* an acute gastro-enteritis may precede the typical enteric condition.

The *mesenteric glands* corresponding to the affected part of the intestine are usually enlarged, sometimes to a very great extent, the whole mesentery being filled with glandular masses. In such glands there may be acute inflammation, and occasionally patchy necrosis occurs. An outstanding feature of the lesions is the extensive proliferation of the endothelial cells along the lymph sinuses and paths, attended by aggregations of mononuclear leucocytes. In other words, the reaction is of the *macrophage* type, and few polymorphonuclear leucocytes are present as a rule. Sometimes on section the glands are of a pale-yellowish colour, the contents being diffuent and consisting largely of leucocytes. Typhoid-paratyphoid bacilli may be isolated both from the glands and the lymphatics connected with them, but *B. coli* is in addition often present.

The *spleen* is enlarged, usually of a fairly firm consistence, of a reddish-pink colour, and in a state of congestion. Of all the solid organs it usually contains the bacilli in greatest numbers. They can be seen in sections, occurring in clumps between the cells, there being no evidence of local reaction round them (Fig. 75). Similar clumps may occur in the *liver* in any situation, and without any local reaction. In this organ, however, there are often small foci of leucocytic infiltration, in which bacilli may not be demonstrable. The bacillus is found, often in large number, in the gall-bladder, where, in recovered cases, it may persist for years (*vide infra*). Clumps of bacilli may also occur in the *kidney*, and localisation of the organism may be observed in the bone marrow.

In addition to these local changes there are also widespread *cellular degenerations in various organs*, which suggest the action of toxic products.

In the *lungs* there may be bronchitis, patches of congestion and of acute bronchopneumonia. In these, the bacilli may sometimes be observed, but evidence of a toxic action depressing the powers of resistance of the lung tissue is found in the fact that the pneumococcus frequently occurs in such complications of enteric fever.

The *nervous system* shows little change, though meningitis associated either with typhoid-paratyphoid bacilli, with *B. coli*, or with streptococci has been observed.

The bacilli have been found in the *roseolar spots* which occur in enteric fever, but it cannot be yet stated that this eruption is always due to the presence of the bacilli.

The fact that the bacilli are usually confined to certain organs and tissues shows that they probably have a selective action.

The reaction of the body to the typhoid-paratyphoid bacilli is markedly one of the lymphoid tissues. This is further indicated by the blood changes, for, while there is a leucopenia, the lymphocytes are relatively increased in numbers. A general reaction is manifested by the appearance of specific antibodies in the serum.

Suppurative lesions occurring in connection with Enteric Fever. In a certain proportion of such lesions the typhoid or paratyphoid bacillus has been the only organism found. This has been the case in subcutaneous abscesses, in suppurative periostitis, suppuration in the parotid, abscesses in the kidneys, empyema, meningitis, etc., and also in a few cases of ulcerative endocarditis. Such suppuration may be of a very chronic and recurrent nature. In the majority of cases other organisms, especially *B. coli* and pyogenic micrococci, have been isolated, typhoid-paratyphoid bacilli being apparently absent. It has, moreover, been shown experimentally that suppuration can be produced by injection in animals, especially rabbits, of pure cultures of the typhoid bacillus, the occurrence of suppuration being favoured by conditions of depressed vitality, etc. It was also found that when typhoid bacilli were injected along with pyogenic staphylococci, the former died out in the pus more quickly than the latter. Accordingly, in clinical cases where the typhoid bacillus is present alone, it is improbable that other organisms have been present at an earlier date.

Occurrence of gall-stones after Enteric Fever. As has been stated, foci of enteric fever, and infection of the gall-bladder, may occur simultaneously.

In the gall-bladder they may set up a catarrhal process (*cholecystitis typhosa*), though frequently they produce comparatively little change in the mucosa. There is evidence that the bacilli may persist in the gall-bladder for many years, and the catarrhal inflammation which they keep up leads to the formation of gall-stones. *B. coli* may be superadded. Typhoid and paratyphoid bacilli have been isolated from the gall-bladder bile, etc., of cases of gall-stones operated on years after an attack of enteric fever, and the bacilli have even been found within the calculi. They have also been demonstrated in chronic suppuration occurring in the gall-bladder.

Typhoid-Paratyphoid Carriers. In the great majority of cases of enteric fever, the bacilli disappear from the faeces within from two to ten weeks of convalescence, but in a certain proportion of cases, probably about 2 to 5 per cent., evidence is found of the persistence of the bacilli for many months, and in certain cases their existence has been demonstrated many years after the attack of illness. Carriers have been arbitrarily classified as 'temporary' (i.e. those excreting bacilli up to a year after an attack of fever) and as 'chronic'. The distinction is unimportant. The former are usually found spontaneously, and the latter are said to be acquired.

The majority of carriers to whom outbreaks have been traced are women. Persons in whom the carrier state is present are a constant danger to those around them, as the infectivity of the bacilli frequently remains, and the importance of such carriers has been recognised as explaining many outbreaks of the disease. The cases traceable to such an origin are of the type usually classed as sporadic. They arise among persons associated with carriers, especially when the latter are concerned in the

great many of these carriers is probably the gall-bladder. As has been stated,

sist there for many years, often
 longer lies in the fact that carriers
 only suffer from slight manifesta-
 —it is well known that in only a

proportion of patients suffering from gall-stones do severe symptoms arise. An additional factor in the carrier problem lies in the fact stated above, that apparently when certain persons ingest the typhoid or paratyphoid bacilli, the latter may establish themselves in the body without giving rise to enteric fever. Such persons have been referred to as 'paradoxical' carriers; they represent those who either are naturally insusceptible to active infection or have developed immunity in consequence of a previous attack. The most serious danger to a community arises, however, from the 'chronic' carrier. In certain carriers the focus of multiplication of the bacilli may be the kidney, and in such cases they are passed out in the urine. Urinary carriers are much less common than biliary carriers; unlike the latter, they occur equally in the two sexes.

The tracing of a carrier constitutes an important and difficult problem. Firstly, the serum of all suspicious persons ought to be submitted to the Widal agglutination test (*vide infra*). Usually the carrier gives a positive reaction, but sometimes agglutination is only obtained with a low dilution of the serum. It has also been shown in chronic carriers that the agglutinating capacity of the serum varies from time to time and sometimes may be absent. Further reference to agglutination reactions in typhoid-paratyphoid carriers will be made later. The proof of a person being a carrier lies essentially in the isolation of the specific organism from the fæces or the urine, and it is to be noted that, especially in the former, the organism is not constantly present—in certain cases months of remission have been recorded. Several explanations have been advanced to account for the facts observed, such as the occurrence of symptomless reinfections or of periodic more or less acute auto-infections from a latent focus of persistence of the organism, e.g. in the gall-bladder. In any case, the necessity for repeated investigation of a suspected carrier is obvious. The methods to be adopted are detailed later. Much work has been directed to the question of freeing the typhoid carrier from the organism, various methods, such as intestinal antiseptics, including treatment with sulphaguanidine, vaccination, excision of the gall-bladder, have been tried. Hitherto success has not been obtained except as a result of operative procedures (excision of the gall-bladder and drainage of the ducts), which appear to have cured a considerable proportion of biliary carriers. In certain urinary carriers examination of the urine from each ureter has shown that the infection is unilateral, being maintained by an abnormal condition such as a calculus in the ureter or kidney pelvis. From the public health standpoint, the prevention of the presence of carriers in a population must be provided for; and in fever hospitals means are taken for retaining convalescents until the bodily discharges are free from the specific bacillus.

The Epidemiology of Enteric Fever. In civilised communities the prevalence of enteric fever has been very markedly reduced, coincident with the substitution of central filtered water supplies for well water and unfiltered river water, and with the improvements effected in general sanitation and especially in the removal of excreta and refuse. In certain localities, however, periodic outbreaks, often of a seasonal character, still occur. At one time these were attributed to the capacity of the typhoid bacillus to live and multiply outside the human body. There is, however, no evidence that the typhoid-paratyphoid bacilli can maintain a saprophytic

existence though they may remain viable in sewage, water, etc., for a variable time. Thus *B. typhosus* and *B. paratyphosus* B have been demonstrated by methods of selective cultivation in the sewage of large communities. In water these organisms survive for at least several days, and even for three or four weeks, the time depending probably on a variety of conditions. Thus typhoid-paratyphoid bacilli tend to persist longer in a relatively pure water than in one grossly polluted and containing large numbers of putrefactive organisms. The existence of carriers in all communities where enteric fever occurs has thrown light, however, on the subject and has accounted for the origin of outbreaks. In many cases survival outside the body for some time is an essential factor where a water or food supply becomes infected with material derived from a carrier, but direct and mediate contact infection also plays a part in the incidence of the disease. At the present time small outbreaks frequently originate in those who are brought into domestic contact with carriers, and larger epi-

quently epidemics due to the paratyphoid *B* bacillus are seldom traced to contaminated water, but are caused by food-stuffs, such as artificial 'cream', in which the bacilli tend to flourish. During epidemics secondary cases may also arise from contact with primary cases.

It is now well known that the house-fly and certain other filth-feeding insects may act as vectors of typhoid and paratyphoid bacilli, and contaminate food to which they gain access after having been in contact with human excreta. Such convection is most marked under conditions of imperfect sanitation, in which human excretal matter is freely exposed to flies, particularly in tropical and subtropical countries and also in the warm season of temperate climates when flies are most numerous. This affords some explanation of the seasonal incidence of enteric fever, its prevalence in ill-sanitated areas and its occurrence under war conditions, particularly in campaigns waged in hot climates. The fly may carry excretal bacteria on the surface of its body, wings, and legs, but typhoid-paratyphoid bacilli may also persist in the alimentary tract of the insect after ingestion of infective excreta (Graham Smith and others). Food is thus contaminated by the insect as a result of its regurgitation and defaecation. In a series of experiments, in which captive flies were artificially infected with typhoid and paratyphoid bacilli and after varying intervals allowed to walk about on a plate of MacConkey's medium which was then incubated, we have found that they remained infective for as long as six days. Colonies of the bacilli developed on the plate in circular groups suggestive of their originating from defaecated or regurgitated material.

Occasional outbreaks of enteric fever and sporadic cases have been traced to oysters and other shellfish, uncooked vegetables such as water-cress, lettuce, etc., contaminated from sewage or human excreta. It has been shown that the typhoid bacillus may persist in living oysters for a considerable time.

Apparently, sometimes animals, *e.g.* cows and dogs, having ingested typhoid-paratyphoid bacilli from human sources, may excrete them for a time and so act as transmitters of these pathogens to man.

The diminution in the incidence of enteric fever in certain countries in recent times, both in war and peace, has been of a remarkable nature. It must be borne in mind that, in addition to immunisation (*vide infra*), the

identification and isolation of carriers and also improvement in general sanitary conditions have been carried out on an extensive scale, and these measures must have contributed to the change in frequency of the disease. How far each of these factors has been individually responsible for the diminution it is not possible to say. It is also noteworthy that in Great Britain there has apparently been a change in the relative prevalence of *B. typhosus* and *B. paratyphosus* *B* infections: at one time *B. typhosus* predominated, but in recent years *B. paratyphosus* *B* has often been responsible for the major proportion of cases of enteric fever.

The isolation of typhoid and paratyphoid bacilli from water supplies and sewage is dealt with in Chapter XXXVII.

IMMUNITY TO TYPHOID-PARATYPHOID BACILLI, AND SEROLOGICAL REACTIONS

Early observers were successful in immunising animals to the typhoid bacillus by repeated injections of small and gradually increasing doses of living cultures of the organism. Later, Brieger, Kitasato and Wassermann found that the bacillus, when modified by being grown in a broth made from an extract of the thymus gland, no longer killed mice and guinea-pigs. These animals after injection were moreover immune, and it was also found that the serum of a guinea-pig thus immunised could, if transferred to another guinea-pig, protect the latter from the subsequent injection of a dose of the typhoid bacillus to which it would naturally succumb. Chantemesse and Vidal, Sanarelli, and also Pfeiffer, immunised guinea-pigs against the subsequent intraperitoneal injection of virulent cultures of the typhoid bacillus, by repeated and gradually increasing intraperitoneal or subcutaneous doses of dead organisms. Experiments performed with serum derived from enteric convalescents indicated that it had *antibacterial* powers, but there was no evidence that it contained any antitoxic bodies (*vide* chapter on Immunity). Pfeiffer, for example, found on adding serum from typhoid convalescents to typhoid bacilli killed by heat, and injected that death took place as in control animals alone. Pfeiffer also found that by using the

could, to a certain extent, protect other animals against the subsequent injection of virulent living bacilli. On trying to use the agent in a curative way, *i.e.* injecting it only after the bacilli had begun to produce their effects, he obtained little or no result. The serum of animals which have been immunised against typhoid and paratyphoid bacilli exhibits various specific antibody reactions *in vitro*: bactericidal action, opsonisation, agglutination, precipitation (with bacterial extracts), and complement-fixation; and the agglutination reaction with specific antisera has been used extensively as a means of identifying these organisms.

Vaccination against Enteric

animals against the typhoid bacillus of enteric fever by Wright and Semple. A killed-culture vaccine of *B. typhosus* was used. During the war of 1914-18, when paratyphoid infections assumed a high prevalence in the Mediterranean campaign, the typhoid-paratyphoid vaccine was introduced, which incorporated killed cultures of the three enteric organisms. This so-called T.A.B. vaccine has since then been universally employed in the prevention of enteric fever in various parts of the world. Sometimes *B. paratyphosus* *C* has also been included in the vaccine. The vaccine has usually been prepared from specially selected

virulent S cultures of the organisms and in the case of *B. paratyphosus B*, cultures in the specific phase (*vide* p. 346). The cultures are emulsified and diluted with normal saline, killed by exposure to 60° C for thirty minutes, and preserved with 0.5 per cent phenol. The vaccine is standardised and the dosage regulated in terms of the number of million organisms per c.c. Two doses at an interval of ten days have generally been administered by subcutaneous injection

	1st dose		2nd dose
<i>B. typhosus</i>	500 millions		1000 millions
<i>B. paratyphosus A</i>	250 or 375	„	500 or 750
<i>B. paratyphosus B</i>	250 or 375	„	500 or 750

The effects of the injection are a variable degree of swelling and tenderness locally and in the regional lymph glands, developing in a few hours, and also a variable amount of general malaise with elevation of temperature, but the local and general effects are transient. During the next ten days the blood

there was also an increase in the capacity of the serum to kill the typhoid bacillus *in vitro*. The second injection usually produces less marked symptoms and is followed by a further increase in the serum agglutinins. These observations indicate that the vaccinated person possesses a degree of immunity to the bacilli, a conclusion borne out by practical results obtained in the use of the vaccine as a prophylactic agent.

During the war of 1914-18 the efficacy of vaccine prophylaxis was put to the test on the most extensive scale. The incidence of enteric fever, an infection which had always been specially prevalent under war conditions, was minimal even in the campaigns waged in tropical and subtropical countries. In the early stages of the Mediterranean campaign, however, enteric fever was prevalent, but the great majority of the cases were paratyphoid infections, and it is significant that the troops were immunised at that time only against the typhoid bacillus. After the introduction of the combined typhoid-paratyphoid vaccine, enteric infections generally were reduced to negligible proportions. Experiences in the last war have amply confirmed the value of the vaccine. Thus, Boyd's observations on the incidence of enteric in allied and enemy troops in North Africa have indicated the efficacy of the British Army T A B vaccine: among several thousand allied prisoners in an enemy camp no cases of enteric fever occurred, though the sanitary conditions were primitive and the disease was occurring in Italian troops in the vicinity. The vaccine used in the Italian army, which was apparently made from non-virulent strains, did not check the prevalence of enteric. The British army vaccine incorporated a strain of *B. typhosus* rich in Vi antigen. It must be remembered that the duration of the artificial immunity is limited, and if it is necessary to maintain a constant prophylaxis immunisation should be repeated at two yearly intervals. It has often been recommended that vaccination should be carried out some time previous to the exposure to infection, according to the view that immediately after inoculation there is a temporary lowering of resistance ('negative phase'). The possibility of such an effect has probably been overestimated.

Recent studies of the antigenic characters of the typhoid paratyphoid bacilli have shown that the full immunising effect of vaccines of these organisms depends on the use of virulent strains representing an adequate content of O antigen and, in the case of the typhoid bacillus, of Vi antigen. The method of preparing the vaccine by heating and preserving it with phenol

is deleterious to the Vi antigen and such vaccine does not lead to the maximum production of Vi antibody. As a result it has recently been advocated by Felix and others that the cultures should be killed and preserved with alcohol, in place of heating and phenolisation; such alcoholised vaccine stimulates the production of typhoid Vi antibody. T.A.B. vaccine of this type is now being used. It apparently produces less severe local and general reactions than the older type. An 'endotoxoid' preparation from the typhoid-paratyphoid bacilli has also been successfully applied for enteric immunisation (Grasset).

Antityphoid Serum. Chantemesse immunised animals with dead cultures of the

successful. Good results have been recorded with an antityphoid serum containing Vi and O antibodies (Felix). It is claimed that the Vi antibody is highly active in promoting phagocytosis of virulent organisms containing the Vi antigen and that it excels the O antibody in this respect. The practical therapeutic value of this serum requires fuller confirmation.

The Serum Diagnosis of Enteric Fever—Widal Reaction. While the most conclusive method of establishing the diagnosis of an enteric infection is the isolation of the causal organism, the indirect proof of the infection by serum diagnosis has been extensively used in routine laboratory work. The method of carrying out this test is referred to in the Appendix. It depends on the presence in the bacillus. The

out on a strictly quantitative basis, so that the highest dilution in which agglutination occurs, i.e. the 'titre', can be determined. Paratyphoid infections have to be considered as well as typhoid, and thus parallel tests with *B. typhosus*, *B. paratyphosus* A and B have generally been carried out in routine practice. Owing to the fact that normal serum in high concentrations may agglutinate these organisms, it is necessary to base the diagnostic interpretation of results on the known range of such 'normal serum effects', i.e. if, however, marked agglutination occurs in a dilution of 1 in 60 with average cultures of *B. typhosus* by the macroscopic method in a person not vaccinated against the typhoid bacillus, the test is considered positive. In the case of a

dilution is significant, may reach higher titres, and with ordinary suspensions of average cultures a titre lower than 1 in 120 cannot be regarded as significant.

All strains do not give uniformly the same results, though it is not definitely known on what this difference of susceptibility depends. A strain must therefore be selected which gives the best results in the greatest number of undoubted cases of enteric fever, and which gives as little reaction as possible with normal sera or sera derived from other febrile diseases. This latter point is important, as some strains react very readily to non-enteric sera. Again, care must be taken as to the state of the culture used. The suitability of a culture may be impaired by varying the conditions of its growth. Standardised bacillary suspensions have therefore been substituted in recent years for suspensions made up from separate cultures, as was previously done on each occasion when Widal reactions were performed. These standardised suspensions were originally preserved with formalin (Dreyer), but this introduced a difficulty. Formalin interferes with O agglutination, though the 'large flake' agglutination characteristic of the H type of antigen is well marked with formalised suspensions. Both O and H agglutinins are formed in enteric infections, but in some cases the agglutinins may be mainly

of the O type, and formalised suspensions would then give negative results. These suspensions are, however, suitable for testing the H agglutinin; and cases occur in which the agglutinins are entirely of the H type. It was found that an analogous standard suspension containing the O antigen alone could be prepared by treating cultures with alcohol, the H antigen being annulled by this method. Felix has advocated the use of specially selected strains which are sensitive to the H and O agglutinins respectively, a formalised suspension of the former and an alcoholised suspension of the latter should be employed. Accordingly, in carrying out the routine Widal test it is now the practice to test the serum in parallel with standard H and O suspensions of *B.*

been found in this country that normal serum agglutination may occasionally reach higher titres, e.g. with *B. typhosus* H suspensions 1 in 80, and with O suspensions, 1 in 100. For determining the titre of O agglutination the mixtures of bacterial suspension and serum dilutions are placed at 50° to 55° C in a water bath and readings made after twenty-four hours; in the cases of H agglutination, readings can be made after two hours at 50° to 55° C.

Standard suspensions for agglutination tests. These are prepared in bulk and either formalised (for H agglutination) or alcoholised (for O agglutination). They maintain their properties for long periods. The fresh batch of suspension is compared with an existing standard in respect of its agglutinability by an appropriate antiserum. The difference observed, if any, is then expressed as a factor by which the titres of sera tested with the suspension can be corrected. In this country such standardised suspensions can be obtained from the Central Public Health Laboratory, Colindale Avenue, London, N.W.9. Marked agglutination without sedimentation is described as standard agglutination. If, for example, standard agglutination results in any test with a suspension up to a certain titre and this suspension is stated to have 'standard' agglutinability, then the titre observed is 'standard titre'. On the other hand if the agglutinability of the suspension is 'standard \times (a certain factor)', the standard titre is found by dividing the dilution-denominator by the factor (see Medical Research Council's Special Report, No. 51). *Formalised suspensions* are prepared by the addition of 0.1 per cent formalin to a twenty-four hours' broth culture or by emulsifying an agar culture in normal saline containing 0.1 per cent formalin. *Alcoholised suspensions* are obtained by emulsifying the growth on 1 in 500 phenol agar in a small amount of saline to which 20 volumes of absolute alcohol are added. The suspension is then heated at 40° to 50° C for thirty minutes. The suspension is finally centrifuged and the deposit re-suspended in saline with chloroform added as a preservative.

The reaction given by the serum in enteric fever usually begins to be observed about the seventh day of the disease, though occasionally it has been found as early as the fifth day, sometimes it may be delayed. Usually it becomes gradually more marked as the disease advances, and it is still given by the blood of convalescents. Further it may persist for several months or longer after recovery. As a rule, up to a certain point, the reaction is more marked where the fever is of a pronounced character, while in the milder cases it is less pronounced, but this is not invariably the case.

Owing to the fact that the development of the specific agglutinins is progressive during the illness, if on the first test agglutination is absent or occurs only in low dilutions within the normal agglutination range, the test should be repeated. In this way a more conclusive result may be obtained.

A series of tests carried out at intervals may elicit a 'rising titre' which is, of course, of the highest diagnostic significance.

The results of the agglutination test are in general relatively specific for the causal typhoid or paratyphoid organism. Thus the serum of a patient infected with *B. paratyphosus A* may agglutinate this organism, but will have little or no effect on *B. typhosus* or *B. paratyphosus B*. These three organisms, however, possess a common O antigenic component and sometimes O agglutination of *B. typhosus* may occur in paratyphoid infection, and O agglutination of a paratyphoid bacillus in true typhoid. As a rule the O agglutination titre is higher for the infecting organism, but the differential diagnosis can best be made by reference to the H agglutination.

It must, of course, be emphasised that such data refer only to persons unvaccinated against these organisms. Further, repeated tests may often be required to elicit conclusive results, and it is well known that in the paratyphoid A infections the agglutinin development is frequently weak or is almost absent till late in the disease.

It should be noted that normal agglutination may vary in degree in different parts of the world and among different communities. It is therefore important that those responsible for diagnostic Widal tests should be acquainted with the range of such normal reactions in the community in which they are working.

The Widal reaction in persons previously inoculated with typhoid-paratyphoid vaccine. Reference has been made above to the limitation of the Widal reaction in persons who have been inoculated with typhoid-paratyphoid vaccine and in whom there is a possibility of either typhoid or paratyphoid infection. In order to surmount the difficulties arising from such complications some observers have used the method of making frequent—it may be daily—estimations during an illness of the highest dilutions in which the serum will agglutinate each of the organisms suspected to be the causal agent. This method was specially elaborated by Dreyer, Amley Walker and Gibson, who held that the study of the curves of the agglutinin content of the serum gave valuable information. Thus, a regular and marked rise in the curve of one of the typhoid-paratyphoid subgroup to a maximum between the sixteenth and twenty-fourth day (especially between the eighteenth and twentieth) with a gradual fall thereafter, indicated an infection with that bacillus, if in such a case primary agglutinins were present towards other members of the group (due to a previous vaccination), the curves of these residual agglutinins either showed no change or a slight rise with a fall to their initial levels, or a marked rise, synchronous with or slightly earlier than that of the curve for the infecting organism. It has also been stated that in persons vaccinated some months previously the residual agglutinins are mainly of the H type, whereas in enteric fever O agglutinins usually appear in the serum and that a diagnosis of enteric in vaccinated persons can be made in this way. Beattie and Elot have found, however, in observations on naval ratings that seven to twelve months after inoculation with typhoid-paratyphoid vaccine the O agglutination with *B. typhosus* and *B. paratyphosus B* may reach a titre of 1 in 160. It is, of course, true that in vaccinated persons the O agglutination titre is usually low and declines rapidly, but a reliable diagnostic result can hardly be obtained by reference to a single O agglutination test. It must be remembered that once agglutinins have resulted from an immunising stimulus non-specific factors, e.g. a non-enteric febrile illness, may increase the agglutination titre of the serum, and an infection by o
a marked increase in

To sum up, in using the Widal reaction for diagnosis in previously vaccinated persons little or no significance can be attached to H agglutination, but if more than six months have elapsed since vaccination, and the O agglutination titre is over 1 in 100 and on repeated testing shows a definite rise, this may be regarded as suggestive of enteric infection.

Vi-agglutination test (*B. typhosus*). If a person has been vaccinated with a heat-killed phenolised vaccine (*vide supra*) and subsequently develops a typhoid infection, the Vi-agglutination test may be of diagnostic value, but repeated tests may be necessary for the demonstration of this agglutinin. The test is best carried out with a selected strain which reacts to the Vi-agglutinin, and is not agglutinated by H and O antibodies. A bacterial suspension for the test can be obtained in concentrated form from the Central Public Health Laboratory. It should not be used after two months owing to its loss of sensitivity when kept. The method of testing generally recommended is to add to 1 c.c. of each serum dilution (1 in 5, 1 in 10, 1 in 20, etc.) in 3 in. by $\frac{1}{2}$ in. tubes, one drop of the concentrated suspension, incubate at 37° C. for two hours and allow the mixtures then to stand

small central compact deposit in the control. In cases of suspected typhoid fever marked agglutination in a titre even of 1 in 10 has been regarded as significant, but of course a rising titre during the illness is more conclusive.

This reaction has now been extensively applied for the recognition of carriers of the typhoid bacillus and has proved a valuable indicator for the purpose.

Strains into types was achieved by Craigie and Yen by adaptation of an anti-Vi phage. When this phage was cultivated serially with different strains, phages were obtained with a high specificity for the strain with which each

types, and a number of new types and subtypes have been added to these (Craigie and Felix). The identification of such types has proved of undoubted value in epidemiological studies, e.g. in tracing the source of an outbreak.

Types of *B. paratyphosus* B. The same principle as that described above has been applied by Felix and Callow to the typing of *B. paratyphosus* B. A heat-labile Vi antigen has been found in every strain examined by them in this country, and by adaptation of an anti-Vi phage to certain strains, different types have been recognised.

Another system of typing has been applied by Kristensen and Boylén and others, based on differences among strains as regards the rate of acid-production in the fermentation of rhamnose and inositol. The stability of the types so recognised is doubtful. For details of the method the original papers should be consulted.

General View of the Relationship of the Typhoid-Paratyphoid Bacilli to Enteric Fever. Enteric fever is a disease in which the lesions are centred in the intestine, but the infection is essentially a general and systemic one, and secondary effects may occur in other parts of the body. The specific association of the typhoid-paratyphoid bacilli with the disease and its characteristic lesions has been thoroughly established by bacteriological

investigation. Their almost constant presence in the blood at an early stage is specially significant of their etiological relationship.

2. The comparative failure of attempts to cause the disease in animals is of little significance. In nature animals do not suffer from this condition and laboratory animals are not susceptible.

3. The fact that serum from enteric patients and convalescents, and the action of such serum in agglutinating the bacilli, indicate an etiological relationship between these organisms and the disease, is a fact that

the disease does arise in those exposed to infection, and also in lowering the mortality when the fever attacks vaccinated persons.

These facts constitute indirect but practically conclusive evidence of the causal relationship of the typhoid-paratyphoid bacilli to the disease. Confirmation of this view is found in the fact that cases have occurred where bacteriologists have accidentally infected themselves by the mouth with pure cultures of the typhoid bacillus, and after the usual incubation period have developed typhoid fever. Several cases of this kind are on record and their significance is not affected by the fact that other similar instances have occurred without the subsequent development of illness. These latter would be accounted for by a low degree of susceptibility on the part of the individual or to a lack of pathogenicity in the cultures.

There is evidence that certain individuals are relatively insusceptible to enteric fever. Cases of the occurrence of typhoid-paratyphoid bacilli in the healthy intestine support this view, and it has been further shown that during an epidemic certain persons may suffer from slight intestinal symptoms with these organisms in the faeces but without the disease going through its usual course. The so-called 'ambulatory' cases of enteric fever form a link between these mild infections and fully developed disease.

BACTERIOLOGICAL DIAGNOSIS OF ENTERIC FEVER

The best solution of this diagnostic problem is found in the isolation of the specific organism from the body of the patient, but difficulty may arise when, as is often the case, the individual does not come under observation till some time has elapsed after the onset of the illness. The bacilli may be isolated in the following ways.

Blood Culture. During the first seven to ten days of the illness—especially in the earlier part of this period—and in relapses the bacilli are present in the blood in most cases and can be demonstrated by blood culture. 5 c.c. of blood drawn from a vein with strict asepsis are placed in 10 c.c. sterile ox bile or in 50 c.c. sterilised broth. The mixture is incubated for 24 hours. Paratyphoid bacilli can be isolated in this way.

Single colony subcultures are made and the organism can then be identified by its biological characters, biochemical and serological reactions.

'Clot culture.' If a sample of clotted blood only is submitted, as for the Widal reaction, it is of value in routine work to use the clot for culture after separation of the serum. For the purpose the clot is transferred directly to a tube of sterilised ox bile and this is then dealt with as in the case of a blood culture (*vide supra*).

Faeces Culture. The method of obtaining suitable samples is referred to in the Appendix. If there is likely to be delay in the sending of specimens to the laboratory, the faeces should be mixed with a sterile solution of 30 per cent.

neutral glycerol in 0.6 per cent. saline buffered with 1 per cent. anhydrous disodium hydrogen phosphate. In the case of a solid or semi-solid stool, a dense emulsion in broth is made and allowed to stand in a tube till the solid particles have settled, one or two loopfuls from the fluid are then used for inoculating plates of *MacConkey's* or *desoxycholate-citrate medium*. The latter is now to be preferred, since it is relatively inhibitory to coliform bacilli and permits a heavier inoculation with the stool, and so increases the chance of isolating the typhoid-paratyphoid organism if the latter is in scanty numbers. Subcultures are made from several discrete pale or colourless colonies on the plate after twenty-four to forty-eight hours' incubation and the pure cultures

cultivated at any stage of the illness.

Brilliant green enrichment method In this method use is made of the property of the dye to inhibit most of the commonly occurring coliform bacilli while allowing the enteric organisms to grow freely. Tubes containing 10 c.c. peptone water (pH 7.0) and (1) 0.25 c.c., (2) 0.1 c.c., and (3) 0.7 c.c. of a 1:10,000 watery solution of the dye are inoculated from the stool and incubated at 37° C. for eighteen hours and then subcultures are made on *MacConkey's* or *desoxycholate-citrate medium*, pale colonies are subcultured and investigated as in other methods. This method gives best results with *B. paratyphosus B.* The procedure may be simplified by using only concentration (2) of brilliant green.

Tetrathionate enrichment The medium consists of 90 c.c. broth to which are added 2.5 grms. of sterilised dried chalk, after thirty minutes steaming of the medium, 10 c.c. of 60 per cent. sterilised crystalline sodium thio-sulphate solution are added and 2 c.c. of iodine solution (6 grms. iodine and 5 grms. potassium iodide in 20 c.c. water). The medium is then tubed in 5 c.c. amounts. A tube is inoculated from the stool and incubated for eighteen to twenty-four hours when a subculture is made on *MacConkey's* or *desoxycholate-citrate medium*.

Sodium selenite enrichment 0.4 per cent. sodium hydrogen selenite (anhydrous) incorporated in a peptone-lactose solution also yields very marked enrichment of typhoid-paratyphoid bacilli from stools (Leifson).

Wilson and Blair's bismuth-sulphite medium. The value of this medium depends on the property of the typhoid-paratyphoid bacilli to reduce sulphite to sulphide in the presence of glucose and the inhibition of coliform bacilli by brilliant green and by bismuth sulphite in the presence of excess of sodium sulphite. Colonies of the enteric organisms growing on the medium are black.

It should be noted that for optimal results more than one of these methods should be used, e.g. direct plating on *desoxycholate-citrate agar* and brilliant green or tetrathionate enrichment. Thus the enteric organism in some cases is demonstrable by one method and not by another.

Urine Culture. The enteric bacilli can be demonstrated in the urine in a certain proportion of cases, especially late in the disease. The specimen of urine is centrifuged and several loopfuls of the deposit inoculated on a plate of *MacConkey's* or *desoxycholate-citrate agar*.

It should be emphasised that many examinations of both stools and urine may be required for the detection of the organism.

Bile Culture. This method has also been applied successfully for diagnosis especially in the later stages of the illness. The duodenal tube is used for aspirating bile and the material is then plated on *MacConkey's* or *desoxycholate-citrate medium*.

Bone Marrow Culture. Recently, sternal puncture has been advocated as a means of obtaining the typhoid or paratyphoid bacillus in cases in which other methods have failed; and successful results have been recorded (Sprenger; Franza and Colarusso).

Widal Reaction. The practical application of serological methods in the diagnosis of enteric fever have been fully discussed in an earlier section of this chapter.

Diagnosis of Typhoid-Paratyphoid Carriers. The agglutination reactions of the serum may be used as a preliminary criterion, but a negative result does not exclude the existence of the carrier state. The only proof is the isolation of a typhoid-paratyphoid organism from the faeces or urine and at least six examinations extending over a period of one month are required, as the excretion of the organisms may be intermittent. It is generally recommended that before the examination the person should receive a dose of calomel followed by a saline purgative and the second or third stool after purgation used for the test. Bile culture is also of value.

Post-mortem Examination. The specific organism may be isolated from the intestine, particularly from ulcerative lesions; cultures may be obtained from the gall-bladder and here the organism may occur in a state of purity. Successful isolation may also be obtained by cultures from the spleen, mesenteric glands, and sometimes the heart blood.

ORGANISMS OF THE *SALMONELLA* GROUP ASSOCIATED WITH FOOD-POISONING, ETC.

Organisms biologically related to the typhoid and paratyphoid bacilli and included along with these in the *Salmonella* group are found associated with cases and outbreaks of acute gastro-enteritis following the ingestion of some article of food, frequently meat, contaminated with the particular organism. Such cases were at one time designated 'ptomaine poisoning', from the idea originally prevailing that the symptoms were caused by ptomaine substances produced from the bacterial decomposition of the proteins of the food. The vast majority of cases of food-poisoning, taking the form of an acute gastro-enteritis, are due to the group of bacteria now under consideration, which are all capable of multiplying in the intestine, they may also produce a general blood infection. (The condition of botulism, a special form of food-poisoning affecting the nervous system, is discussed in Chapter XX.) The food at fault may not, to taste or smell, present any unusual features, but very often there can be isolated from it an organism identical with that derived from the sick individuals. The foods giving rise to poisoning usually belong to the preserved-food class, e.g. sausages or similar products, but cases also arise from fresh foods, milk, etc. Sometimes it has been proved that the animal from which the food is derived has been suffering from infection due to the organism subsequently found, but this has not always been the case, and meat from healthy animals may be contaminated from extraneous sources.

BACILLUS ENTERITIDIS (*SALMONELLA ENTERITIDIS*), ETC.

The classical type of the organisms referred to above is *B. enteritidis* described first by Gaertner (1888) in an outbreak of gastro-enteritis following the ingestion of the flesh of a diseased cow. The organism was isolated both from the patients and from the meat. Subsequent similar outbreaks in various countries were found to be due to the same organism which was demon-

strated in the stools and organs of cases. This organism resembles *B. paratyphosus* B in its general characters, including biochemical reactions (*vide* Table, p. 362), but can be clearly differentiated by its serological characters. The *Salmon-*
or laboratory
ense hæmor-
 rhagic enteritis, and usually there is a septicæmia with the occurrence of serous inflammations; the bacilli are recoverable from the internal organs and often from the blood. In man, as the name of the bacillus indicates, the effects are centred in the intestine, where there is usually marked inflammation of the mucous membrane, sometimes attended with hæmorrhage into it; evidence of a septicæmic condition may also exist. The illness usually appears within twenty-four hours of the food being taken, but in some cases symptoms may appear almost at once, in which case they are probably due to the immediate action of toxin; it is important to note that the poisons formed by this type of organism are relatively heat-resisting. The production of meningitis and cholecystitis along with enteritis, has been described in infants by Guthrie and Montgomery. The serum of convalescents may agglutinate the organism.

Danysz's bacillus and rat viruses. Danysz isolated from an epizootic in field mice an organism which is practically identical with *B. enteritidis*, and introduced it for the purpose of killing rats by originating in them through feeding a similar epizootic. Several 'viruses' of this kind have been in commercial use for this purpose, e.g. 'Ratin'. The efficacy of such agents may vary, and the mortality in artificially originated epizootics may be from 20 to 50 per cent. Sometimes, apparently under natural conditions, rats develop an immunity to such 'viruses'. This type of organism may be regarded as a variety of *B. enteritidis* and designated *danysz*; it shows minor fermentative differences from the classical type. (Another variety designated *essen* was isolated from enteritis in man and from ducks and ducks' eggs, a further variety designated *chaco* was obtained from cases of continued fever.)

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 bee
 typ

Salmonella group considered as a whole comprises a multiplicity of types resembling one another in general and biochemical characters but differing in antigenic constitution and in particular biochemical reactions. In a recent publication in which these organisms are comprehensively described, 111 types are listed (Wilson and Miles). Their serological classification is dealt with later.

Savage and White in a study of the organisms of this group associated with food-poisoning, recognised in 1925 certain main types: *B. enteritidis* (Gaertner), *B. aertrycke*, *B. suispestifer*, and other varieties designated 'Stanley', 'Newport', 'Derby'. Since then the *Salmonella* group has received most intensive study and as a result the number of recognised types has steadily grown. Their importance lies not only in their relationship to food-poisoning, but also in their pathogenic rôle in various animals.

B. aertrycke (*Salmonella typhi-murium*) was originally isolated by De
B. suispesti-
viously been
hog cholera)

which, however, was later shown to be due to a filterable virus, *B. suispestifer* being probably a secondary agent in the disease. The biochemical reactions of these organisms are shown in the Table on p. 362, and their antigenic characters in the Table on pp. 317-8.

They were at one time confused with *B. paratyphosus B* owing to their close serological relationship with it, and similarly strains designated *B. paratyphosus B* were described in food-poisoning cases. Savage and White separated them into the different types referred to above, and pointed out that *B. paratyphosus B* is associated with paratyphoid fever and probably enteric fever due to the typhoid bacillus, while *B. paratyphosus B* usher in the attack in food-poisoning.

though *B. aertrycke* is a frequent cause. All recent data on the occurrence of *Salmonella* group organisms in human infections in this country indicate that *B. aertrycke* is by far the most prevalent. Other types reported up to 1939 were *B. enteritidis*, Thompson, Newport, *B. suispestifer* (diphasic and monophasic varieties), *B. moribificans bovis*, Dublin, Senftenberg var. Newcastle, Derby, Stanley, Eastbourne, Potsdam, Aberdeen, and London. The antigenic characters of these organisms are dealt with below and their biochemical reactions are indicated on p. 362. In 1912 a number of new types were isolated in this country, the commonest being the Oranienburg, and since then still further types have been recognised, mostly varieties previously described in the United States and other countries. It seems likely that many of these were introduced in imported dried egg. In addition to the Oranienburg type those which have assumed some degree of prevalence are *S. anatum*, *S. melagroidis*, Bareilly, Chester, Tennessee, and Montevideo types. In all, twenty-nine additional types were isolated between 1940 and 1944, and many strains were unidentified.

Smith has emphasised the variation in the form of disease produced by *Salmonella* types *B. aertrycke*, *B. enteritidis*, and the Thompson type are usually responsible for an enteritis, while the Dublin type has been associated with septicæmia and meningitis. *B. suispestifer* also seems to possess considerable invasive properties. Cases have been recorded which at first had the symptoms of food-poisoning and later developed into a continued fever.

In the etiology of food-poisoning the sources of the causal organisms and the mode of contamination of the offending article of food require consideration. *B. enteritidis* or varieties of this organism occur in natural infections in cows, calves, pigs, ducks, rats, and mice. *B. aertrycke* is responsible for epizootic enteritis in guinea-pigs, mice, and other rodents, and is found in pigs, but not commonly in rats (Savage and White). It may occur also in cattle, sheep, pigeons, ducks, chickens, parrots, and canaries. Thus, in the case of flesh foods, the animal from which the food is derived may have been infected, e.g. the original *B. enteritidis* strains. As noted above, preserved foods have frequently been responsible for outbreaks, and it has been assumed that growth of the particular organism may have occurred in the material with the formation of toxin which, on ingestion of the food, is responsible for immediate symptoms. Cow's milk has on occasions been responsible for outbreaks of food-poisoning, the organism being derived from the udder or intestinal tract. As the *Salmonella* organisms are prevalent in mice and rats, contamination of food may occur from these animals. Eggs from infected birds, e.g. ducks, may contain the organism, and food-poisoning has in some cases been traced to this source. Reference has already been made to *Salmonella* infections from imported dried egg. In certain instances, human carriers of these organisms may be responsible for food contamination and outbreaks of enteritis following the ingestion of the food.

Toxic and antigenic chemical fractions isolated from *Salmonella* group organisms. Studies of the chemical fractions isolated from *B. aertrycke* and *B. enteritidis* have been made by Boivin, Raistrick and Topley, and others.

By extraction of the bacterial cells and purification of the resulting products a protein-free fraction has been obtained consisting of a complex carbohydrate combined with phosphatide. This apparently constitutes the endotoxin and also the somatic antigen of the organism. It is highly toxic, and animals injected with it develop hyperglycæmia. *In vivo* it incites the production of somatic agglutinins. It has been suggested that this type of chemical substance isolated from *Salmonella* organisms is representative of similar toxic and antigenic products common to various bacteria, these differing chemically from one another according to the species or type of organism.

Bacteriological Diagnosis of Salmonella Food-poisoning. Cultures should be made from the stools on plates of MacConkey's or desoxycholate-citrate medium. Separate pale colonies are subcultured, and the cultures are investigated first in regard to their biochemical characters and serological reactions. In addition to the ordinary fermentation tests with sugars and similar carbohydrates shown in the Table on p. 362, certain other biochemical reactions have been utilised for differential purposes. Among these, the utilisation of sodium *d*-tartrate merits consideration: thus *B. aertrycke* and various other *Salmonella* group organisms generally utilise this salt while strains of *B. paratyphosus B* from enteric fever have no action on it (Brown, Duncan and Henry). On the other hand, it has been pointed out that strains of *B. paratyphosus B* from cases of gastro-enteritis utilise the salt. To test the difference the organisms are grown in peptone water containing 1 per cent. of the salt. After incubation a solution of lead acetate is added to the culture: if the salt has not been acted on there is a copious precipitate of an insoluble lead compound with the organic acid; utilisation is indicated by absence of such precipitation though a slight precipitate of carbonate may be observed. Indole production is uniformly absent in the *Salmonella* group except for occasional variant strains or colonies. Gelatin liquefaction is also absent but liquefying types have been recorded. The serological identification of these organisms is considered below. In certain cases, particularly those of a severe nature, the causal organism is present in the blood and can be isolated by blood culture. The suspected article of food, if obtainable, should be examined bacteriologically with a view to isolating the causal organism from it.

In convalescence, if a bacteriological diagnosis has not previously been made, examination of the patient's serum by direct agglutination tests and agglutinin absorption tests with known *Salmonella* strains of different types may elicit proof of the previous infection. The serum is tested with specific H suspensions of *B. aertrycke*, *B. paratyphosus C*, *B. enteritidis*, *Newport*, and *Stanley* types. These are obtainable from the Central Public Health Laboratory, London. A reaction with one of them in titres above 1 in 50 is regarded as significant. A mixed suspension consisting of mono-phasic non-specific *aertrycke* and *suspestifer* types can also be used, but of course a reaction will merely denote infection by a member of the *Salmonella* group; a titre of over 1 in 50 would be suggestive. Previous enteric vaccination would nullify the significance of all these reactions.

THE SEROLOGICAL CLASSIFICATION OF THE SALMONELLA GROUP

In recent years the antigenic composition of the *Salmonella* group has been studied by various workers (notably by Kauffmann and Bruce White) and an attempt has been made to define in this way the serological differences

and inter-relationships of the various types. Reference has been made to the close relationship between different members of the group and the difficulty of differentiating such organisms by simple agglutination tests with specific antisera. The occurrence of such cross-agglutination is due to the fact that an organism not only stimulates the production of agglutinins towards itself (homologous), but also of agglutinins acting on kindred species (heterologous, group agglutinins, or co-agglutinins). The homologous agglutinins may be present in much greater amount in the serum than the heterologous, and careful quantitative tests would elicit this difference; but among certain organisms group agglutination may be so marked as to render direct agglutination tests insufficient for differentiation.

In differentiating between homologous and group agglutinins the absorption method has been used. Thus, Castellani found that, when an animal had been immunised with *B. typhosus*, this organism would *in vitro* remove ('absorb') from the serum not only the homologous typhoid agglutinins, but also the group agglutinins which might act on allied organisms, whereas *in vitro* treatment with a related organism removed only the group agglutinins for that organism without influencing the typhoid agglutinins.

The occurrence of group agglutinins in the serum of an animal immunised against a particular organism is due at basis to the complexity of the antigenic structure of the bacillus in question, some antigens being specific or peculiar to the bacillus, others being possessed in common with other members of the group. It was at one time assumed that the individual bacilli in a pure culture all possessed the same antigenic structure. The work of Andrewes, however, showed that in the case of certain members of the *Salmonella* group the two kinds of antigen are carried by different bacilli and their derivative colonies in plate cultures. These organisms are therefore described as 'diphasic'. In analysing the subject, Andrewes first prepared a pure specific serum and a pure group serum. The former was obtained by absorbing the group agglutinins in an antiserum for a particular organism by treatment with an emulsion of another bacillus of the same group. The group serum was simply a serum prepared against another bacillus of the group, provided that it was rich in group agglutinins. When agar plates were prepared and individual colonies were separately tested, it was found that the bacilli of some colonies were agglutinated by the specific serum, but practically not at all by the group serum; while the converse held in the case of other colonies. In the mass culture there are accordingly two kinds of colony differing in their

kind, the other soon re-
character is entirely sta-

H antigen. Thus, in one of these phases this antigen is specific, in the other it exhibits well-marked group characters. It was found later that in some cases the diphasic variation does not involve a difference between specific and group characters, and the designation of the alternative phases as 'specific' and 'group' has not proved generally applicable. It has therefore become customary now to refer to them simply as Phase 1 and Phase 2, but it may be said that Phase 1 often represents the specific antigens, and Phase 2 the non-specific.

Apart from the H antigenic components common to different types in one phase, there may also be common O constituents among certain organisms. Thus, the whole group can be arranged in subgroups according to

similarities in their O antigens. These relationships are illustrated in the Table (*vide infra*). This shows the main antigenic factors of several representative types, and illustrates the inter-relationships dependent on the existence of factors common to two or more different organisms of the group. It should be noted that the various antigenic formulæ shown in the table do not necessarily represent the complete antigenic constitution of the respective types and that components specified by a single number or letter may each be of complex structure.

SEROLOGICAL IDENTIFICATION OF SALMONELLA TYPES

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sera

G,

the Kauffmann-White classification. A detailed description of the method is given in the Bulletin of the Ministry of Health, etc., 1944, 3, 177.

pension of the unknown strain, if large flake agglutination results the organism is probably in the group phase and from it a specific-phase derivative must be separated. This can often be secured by plating, so as to obtain separate colonies. A loopful from each of a number of colonies is emulsified on a slide in a drop of antiserum (in low dilution) to the monophasic *B. suspestifer* referred to above. The presence or absence

confirmed by absorption tests. A non-motile variant can only be identified serologically as regards its O antigen. For fuller information the original papers on this subject should be consulted. The Central Public Health Laboratory supplies a certain range of antisera for identifying *Salmonella* types, details of these and their practical use are given in the *Bulletin of the Ministry of Health*, etc., 1944, 3, 177. For the detailed identification of the antigenic components of an unknown *Salmonella* type a full antigenic analysis is made by means of selectively absorbed monospecific sera for the various O and H components of the group (*vide Table*.)

REPRESENTATIVES OF THE SALMONELLA GROUP

Arranged in subgroups with similar O antigens according to the Kauffmann-White classification

SUB-GROUP	TYPE	O (somatic) ANTIGEN	H (flagellar) ANTIGEN	
			Phase 1	Phase 2
A	<i>paratyphosus A</i>	(I), II, XII...	a	—
B	<i>paratyphosus B</i>	(I), IV, (V), XII...	b	1, 2...
	<i>enteritidis (typhi-murium)</i>	(I), IV, (V), XII...	c	1, 2, 3...
	<i>Stanley</i>	IV, V, XII...	d	1, 2...
	<i>Chester</i>	IV (V), XII...	e, h	e, n, x...
	<i>Realing</i>	IV, XII...	e, h	1, 5...
	<i>Derby</i>	(I), IV, XII...	f, g	—
	<i>abortus equi</i>	IV, XII...	—	e, n, x...
	<i>abortus ovis</i>	IV, XII...	c	1, 6...
	<i>abortus bovis</i>	(I), IV, XXVII, XII	b	e, n, x

REPRESENTATIVES OF THE SALMONELLA GROUP—continued

SUB-GROUP.	TYPE	O (somatic) ANTIGEN.	H (flagellar) ANTIGEN.	
			Phase 1.	Phase 2
C	<i>paratyphosus C</i>	VI ₁ , VI ₂ , VII... (Vi)	c	1, 5 ..
	<i>suispestifer</i> * (<i>cholerae-suis</i>)	VI ₁ or 2, VII...	c	1, 5...
	<i>typhi-suis</i>	VI ₁ , VI ₂ , VII...	c	1, 5
	Thompson	VI ₁ , VI ₂ , VII...	k	1, 5...
	Oranienburg	VI ₁ , VI ₂ , VII...	m, t	—
	Potsdam	VI ₁ , VI ₂ , VII...	l, v...	e, n, z ₃₁
	Bareilly	VI ₁ , VI ₂ , VII...	y	1, 5.
	Montevideo 1	VI ₁ , VI ₂ , VII...	g, m, s	—
	Montevideo 2	VI ₁ , VI ₂ , VII...	g, m, s.	—
	Tennessee	VI ₁ , VII...	z ₂₉	—
	Newport	VI ₁ , VIII...	e, h	1, 2, 3 ..
D	<i>morbificans bovis</i>	VI ₁ , VIII...	r	1, 5.
	<i>typhosus</i>	IX, XII... (Vi)	d	—
	<i>enteritidis</i>	(I), IX, XII...	g, m	—
	Dublin	I, IX, XII...	g, p	—
	Eastbourne	(I), IX, XII...	e, h	1, 5...
	Senda	(I), IX, XII...	a	1, 5.
	<i>gallinarum</i>	IX, XII...	—	—
E	<i>pullorum</i>	IX, XII...	—	—
	London	III, X, XXVI	l, v ..	1, 6...
	anatum	III, X, XXVI	e, h...	1, 6...
	meleagridis	III, X, XXVI	e, h	1, w
	Newington	III, XV	e, h	1, 6.
	Senftenberg	I, III, XIX	g, s, t ..	—
Other subgroups	Aberdeen	XI	i	1, 2, 3.
	Poona	XIII, XXII	z...	1, 6.
	Florida	(I), VI, XIV, XXV	d	1, 7
	gamunira	XVI	d	1, 7
	Kirkee	XVII	b	1, 2.
	Cerro	XVIII	z ₄ , z ₂₃ , z ₂₅	—
	Kentucky	(VIII), XX	i	z ₄ .
	Minnesota	XXI, XXVI	b	e, n, x.
	Tel-aviv	XXVIII	y	e, n, z ₁₅ ...
	Ballerup	XXIX (Vi)	z ₁₄	—
	urbana	XXX	b	e, n, x.
	Arizona	XXXIII	z ₄ , z ₂₃ , z ₂₆	—
	Adelaide	XXXV	f, g	—
	Inverness	XXXVIII	k	1, 6.

* A monophasic (Phase 2) variety has been described and designated 'Kunzendorf'

See Classification of Salmonella Subcommittee of the International Society of Microbiology, *J. Hyg.*, 1934, 34, 333, Kauffmann, *Zt. f. Hyg.*, 193" 120, 177, 'Die Bakteriologie der Salmonella-Gruppe', 1941, Copenhagen, Bornstein, *J. Immun.*, 1943 46, 439, and Wilson and Miles, 'Principles of Bacteriology and Immunity', 1946, London, 1, 713

In this classification, different O antigenic constituents are represented by Roman numerals, H components (Phase 1) by small letters and H components (Phase 2) by

Subgroup A.

B paratyphosus A stands in a subgroup by itself, but has an H antigen ('a') in common with several other types, e.g. *Sendai*. It is monophasic.

Subgroup B.

B paratyphosus B is assigned to subgroup B, which includes a large number of types with the common O antigens 'IV' and 'XII'. This organism is diphasic with the 'b' flagellar component in the specific phase.

B aertrycke (*S typhi-murium*) has similar O constituents to those of the former organism, but in the specific phase has an entirely different flagellar antigen ('1'), in the non-specific phase it cannot be distinguished from *B paratyphosus B* by serological methods.

Stanley type This has been found in food-poisoning cases, but is unknown so far in animal disease. Recently, however, it has been isolated from American dried egg imported into this country. It is related through its O antigens to *B paratyphosus B*, but has a distinctive H antigen in the specific phase.

Chester type has been isolated from gastro-enteritis in various parts of the world and has been found in healthy pigs. It is diphasic, one phase having the H antigens 'e', 'h', and the other 'e', 'n', and 'x'.

Reading type, originally isolated from a water supply and later from gastro-enteritis and healthy human carriers. It has been found in healthy pigs and from an epizootic in guinea-pigs. In the specific phase it is similar to one phase of the *Chester* type.

Derby type has been found in gastro-enteritis and in healthy pigs, turkeys, and imported dried egg. The flagellar antigens are different from those of the other members of the subgroup. It is apparently monophasic.

B abortus-equi This occurs in abortion in mares, and has not been found in human infections. Though naturally monophasic, artificial antigenic variation has been observed in this organism: one such variant has been found to possess the 'a' component (cf. *B paratyphosus A*).

B abortus-ovis described in abortion of sheep, it is diphasic, and in the specific phase has the same flagellar antigen as *B suspestifer*.

B abortus-bovis, isolated from the viscera of cows that had aborted and also found in a case of enteric-like illness. In one phase it has the same H antigen ('b') as *B paratyphosus B*; in the other phase the H antigen resembles that of *B abortus-equi* and *B Chester*. It is exceptional in its property of liquefying gelatin.

Subgroup C.

B paratyphosus C (Hirschfeld type of *Salmonella*) has already been referred to. It has been reported in enteric cases in Eastern Europe and British Guiana, but has not been isolated from animals. Its antigenic structure is similar to that of *B suspestifer* (*S cholerae-suis*) and like the latter it is diphasic. *B paratyphosus C* and *B suspestifer* are distinguishable by biochemical reactions (vide Table, p. 362).

B suspestifer (*S cholerae-suis*) Reference has already been made to the occurrence of this organism in pigs and its occasional association with gastro-enteritis in man. It has been found in cattle and in imported dried egg. Two varieties have now been recognised, one with the VI₁ somatic antigen and the other VI₂, both being diphasic and otherwise similar. At one time a monophasic variety which seemed to exist only in the non-specific phase was described as the *Kuntendorf* type: this variety differing from the classical strains in the production of H₂S. It is now doubtful whether this supposed variety should be separately named, as it can be transformed under certain conditions to the specific phase. It has been pointed out, however, that it differs in pathogenicity from the typical strains of *B suspestifer*, being less invasive. Enteric-like infections by *B suspestifer* and the *Kuntendorf* type have been recorded.

Salmonella typhi-suis originally isolated from a typhoid-like disease of pigs, seems to be associated with infection in these animals alone: it has not been found in human disease. It is practically identical with *B suspestifer* in antigenic structure but differs in its biochemical reactions (vide Table, p. 362).

Thompson type This has been frequently isolated from food poisoning and has a relatively high prevalence in this country. It has been found also in pigs and has been isolated from imported dried egg. It is diphasic. The specific antigen ('k') distinguishes it from *B suspestifer*.

Oranienburg type This organism occurs in gastro-enteritis and infantile diarrhoea. It has been found in chickens in the United States and has frequently been isolated from imported dried egg.

Potdam type occurs in food poisoning and has recently been found in imported dried egg.

It has been found in this country have resulted from its presence.

Montevideo type. Two varieties have been recognised, isolated from pigs, chickens, and turkeys, and from imported dried egg, and has been responsible for outbreaks of food-poisoning in this country.

Tennessee type has been reported recently in cases of food-poisoning originating from imported dried egg.

Newport type. This has been one of the commonest food-poisoning organisms in this country. It has been found in rats, pigs, and poultry. Imported dried egg has also been found to contain it.

B. moribundus *bovis* has been isolated from infected cattle, though relatively uncommon, and from cases of gastro-enteritis in man. It has also been found in dried egg.

Subgroup D.

B. typhosus. This organism has already been described in detail. It has the O antigens 'IX' and 'XII' and, when recently isolated, strains have the 'Vi' antigen. It is monophasic and possesses the specific flagellar antigen 'd' which it shares with several *Salmonella* types, e.g. *Stanley*, belonging to other subgroups. It has not been found in animals.

B. enteritidis is similar in its O antigenic structure to *B. typhosus*, but differs in its H antigens, it is also monophasic. The characters of the organism and its relation to food-poisoning have already been dealt with.

Dublin type. As the Table shows, this organism has a close relationship to *B. enteritidis*, though it has a different component ('p') in Phase 1. It has sometimes been described as a variety of *B. enteritidis*. It occurs in calves, e.g. calf diarrhoea, and less commonly in adult cattle, but it also occurs in fowls and pigs. It has been found in gastro-enteritis of man and has been responsible for milk-borne outbreaks.

Eastbourne type. This was first isolated from a case diagnosed as enteric fever. It has also been found in animals. Certain strains differ from other *Salmonella* organisms in producing indole.

Sendai type was first described in enteric fever in Japan and has also been met with in America. Though similar in its O antigens to *B. typhosus*, it resembles *B. paratyphosus* A in its H antigen. It is, however, diphasic.

B. gallinarum occurs in a disease of fowls sometimes spoken of as 'fowl typhoid'. It differs from other members of the *Salmonella* group described above in being non-motile and non-flagellate and therefore possessing only O antigens. It is practically non-gas-producing. The biochemical reactions are shown in the Table on p. 382. This organism is generally regarded as non-pathogenic to man, but a fermentative variant has been found in cases of gastro-enteritis.

B. pullorum is responsible for an exceedingly common disease of chickens known as 'bacillary white diarrhoea'. It can be isolated from the faeces, internal organs, and blood, and the infection is transmitted through the egg from the parent bird. Adult hens may carry the organism without obvious signs of infection, such carriers can be recognised by the agglutination reaction of their serum with a culture of *B. pullorum*. This organism has sometimes been found in other birds, in pigs, and in the human subject, and has been isolated from imported dried egg. It is apparently identical in antigenic structure with *B. gallinarum* and is likewise non-flagellate, but differs in biochemical characters (vide Table p. 382), being usually gas-producing and failing to ferment ducitol or to utilise d-tartrate. Some workers have regarded *B. pullorum* as a fermentative variant of *B. gallinarum*.

Subgroup E.

London type has been isolated from food-poisoning and infantile diarrhoea. It has also been found in healthy pigs, chickens, and imported dried egg. It has the O antigens 'III', 'X', and 'XXVI', and is diphasic, the specific phase antigens being '1', 'v', 'III', 'X', and 'XXVI', and is diphasic, the specific phase antigens being '1', 'v', 'III', 'X', and 'XXVI'.

Salmonella anatum is similar in its O antigens to the *London* type, but with different H antigens ('e', 'h'). It was originally described in an epizootic intestinal infection of ducklings ('keel disease'), and has been isolated from cases of food-poisoning and infantile diarrhoea. It has been found in imported dried egg.

Salmonella meleagridis. This type is one of those which have been isolated recently in this country from imported dried egg. It is associated with infections in turkeys and has been isolated from pigs. It is similar to *S. anatum* in its O antigens and also its H antigens in one phase, but different in respect of the H components of the other phase.

Newington type isolated from ducklings, chickens, turkeys, and dried egg imported into Great Britain.

Senftenberg type isolated originally from a case of gastro-enteritis. It has been found in turkeys and chickens and in healthy pigs.

Other subgroups.

Aberdeen type was originally isolated from a case of gastro-enteritis, it has also been found in chickens.

Some further representative types which fall into subgroups other than A to E are shown in the Table, and their differential antigenic formulæ are given. Most of them have been found in cases of gastro-enteritis and some have been isolated from poultry and pigs. One type, *Arizona*, has been isolated in the United States from certain reptiles.

CHAPTER XV

THE DYSENTERY BACILLI AND ALLIED ORGANISMS

DYSENTERY—a clinical term applied to cases characterised by tenesmus and the frequent passing of stools containing blood and mucus—has long been recognised as including several etiologically distinct conditions. There are two main forms of dysentery—*amœbic* due to the *Entamoeba histolytica* (Chapter XXXIV), and *bacillary*, due to *B. dysenteriae*, a group of organisms comprising a number of different types. In both cases the lesions are situated in the large intestine. The dysentery bacilli are related biologically to the colityphoid organisms. It should be noted that cases with the clinical features of food-poisoning may sometimes be due to infection with dysentery bacilli.

The first organism of this group to be described is that known as *B. dysenteriae*, Shiga type, observed by Shiga (1898) in cases of bacillary dysentery in Japan. Subsequently workers in different parts of the world confirmed Shiga's observations (Kruse and others); and similar organisms differing from the Shiga type in certain biological characters were also described (Flexner, and Hiss and Russell). It was further recognised that epidemics of dysentery occurring from time to time in mental hospitals were due to this group, and that the same organisms were responsible for cases of acute enteritis in infants and children—the so-called 'summer diarrhoea'. Important contributions were made to the subject of bacillary dysentery during the war of 1914-18, when this condition assumed serious proportions among troops, especially in those campaigns carried on in tropical and sub-tropical areas, and new types of dysentery bacilli were described. Since then still further extensions have been made to our knowledge of the group of dysentery bacilli. The evidence for the causal relationship of these organisms consists chiefly in their constant presence in the dejecta in the early stage of the illness and in the agglutination of the associated strains by the serum of patients, but confirmatory evidence has also come from animal experiments. While different biological types of *B. dysenteriae* are recognised, they all present certain common characters and constitute a fairly well-defined group. The differentiation of types depends on differences in fermentation and biochemical reactions and in serological characters.

B. DYSENTERIÆ, SHIGA TYPE (*SHIGELLA DYSENTERIÆ*) and B. DYSENTERIÆ, FLEXNER TYPE (*SHIGELLA PARADYSENTERIÆ*)

These constitute the classical members of the group

MICROSCOPIC CHARACTERS. The bacilli morphologically resemble the typhoid bacillus but differ from it in being non-motile and non-flagellate. They sometimes show a tendency to the cocco-bacillary form. No spore formation occurs. They are stained readily by the ordinary dyes, and are Gram-negative.

CULTURAL CHARACTERS. Conditions for growth are similar to those of the typhoid bacillus. On nutrient agar the colonies also resemble those of the typhoid bacillus, being usually of smaller size and less opaque than those of the *B. coli*. 'Rough' colony variants may be observed in laboratory cultures, and sometimes a variant characterised by 'mucoid' colonies (Fletcher). There is still some doubt whether the rough variant is analogous

in antigenic characters to the rough variants of the *Salmonella* group, thus the antigenic change associated with this colonial variation seems to be of a specific \rightarrow group nature (*vide infra*), not the usual S \rightarrow R transformation. In *gelatin stab* culture no liquefaction results. On *MacConkey's* and *desoxycholate-citrate medium* the colonies are pale and colourless. In *broth* a uniform turbidity is produced. In *litmus milk* there is developed at first a slight degree of acidity, which is followed, in the case of certain strains of dysentery bacilli, by a phase of increased alkalinity, no coagulation of the milk occurs. On *potato* the organism forms a transparent or whitish layer, which, however, in the course of a few days assumes a dirty grey colour, with some discoloration of the potato at the margin of the growth.

DIFFERENTIATION OF SHIGA AND FLEXNER TYPES. As has been indicated, the two types behave differently in biochemical and serological reactions. They both ferment glucose without gas production, and do not ferment lactose. In all fermentation reactions they are non-gas-producing (*vide* Table, p. 362). The Shiga type has no action on saccharose, mannitol, maltose, or dulcitol, and does not produce indole in peptone water. The type originally described by Flexner differs from the Shiga type in the fermentation of mannitol and maltose, and in the production of indole. Saccharose fermentation by some strains of the Flexner type after laboratory cultivation has been recorded. Hiss and Russell described strains similar to the Flexner type, but differing in the absence of maltose fermentation, and designated these the 'Y' type. Thus the typical dysentery bacilli were differentiated into two main types—the 'non-mannitol-fermenters' (Shiga) and the 'mannitol-fermenters' (Flexner-Y), but strains of the latter are not homogeneous either in biochemical reactions or in serological characters. These mannitol-fermenting strains vary in the production of indole and the fermentation of maltose. Indole production even in the same strain may be variable. It is customary now to designate all strains corresponding to the classical Flexner and Y organisms as of the 'Flexner type'. As a further

antiserum to the Flexner type (*vide infra*)

SEROLOGY OF THE FLEXNER TYPE. It was pointed out by Andrewes and Inman that the organisms of the Flexner type possess four different antigenic components designated V, W, X, Z, one of which may predominate on this Y, and They regarded the Y race as presenting a mixture of the V, W, X, and Z components more evenly balanced than in the other races. Thus an anti-Y serum has a wider agglutination range than sera for other strains. Two sub-races VZ and WX were also described.

More recent studies of the serological characters of these organisms have thrown further light on their antigenic structure and inter-relations. According to Boyd strains normally possess a common or group antigen, but are differentiated into six types (or subtypes) each with a distinctive specific antigen. These include the V, W, and Z subtypes of Andrewes and Inman and three others corresponding to Boyd's strains '103', 'P119', and '88'. They may conveniently be designated I, II, VI respectively. As a result

would explain its representative serological character. There is some doubt

whether the X subtype of Andrewes and Inman is a distinctive variety, Boyd suggests it is a variant of Z. It may also be noted here that subtype VI ('88') is serologically identical with the organism called the Newcastle dysentery bacillus, though it has the biochemical characters of the Flexner type. Boyd also found that certain dysentery bacilli (isolated in India) though corresponding in biochemical reactions to the Flexner type differed from this organism in the absence of the common Flexner antigen and he recognised three such types with distinctive serological characters corresponding to his strains '170', 'P288', and 'D1'. These have been designated 'Boyd India' types I, II, and III respectively, but whether they should be separated from the Flexner type is open to question. Wheeler has extended the number of subtypes of the Flexner organism to nine, and Weil and others, who group together Boyd's subtypes of the Flexner organism and those designated 'India', have extended the total number of all such subtypes to fourteen. Boyd has also recognised additional types of the serological subgroup to which he assigned the types originally named 'India'; and experience of bacillary dysentery during the late war has further shown that all these types have a wide geographical distribution. Thus, the dysentery bacilli with the cultural reactions of the Flexner type show a marked degree of heterogeneity in their antigenic constitution and are also subject to antigenic variation. The Shiga type on the other hand is definitely homogeneous in this respect. It is of interest that antisera for the Shiga type may agglutinate some strains of the Flexner type. Some strains of the Flexner type possess *Salmonella* group antigens.

The characters of the various other types of dysentery bacilli are dealt with later (p. 357).

VIABILITY In general the dysentery bacilli correspond to the typhoid bacillus in their viability. Cultures are killed at 55° C. within an hour. In stools kept at ordinary temperature the organisms do not survive longer than twenty-four hours and may die in seven or eight hours. Cultures which have been dried and exposed to light die in six to twenty-four hours. The duration of survival in water, as in the case of the typhoid bacillus, depends on various factors, including the degree of pollution of the water; thus, in a grossly polluted water dysentery bacilli may not survive longer than forty-eight hours.

Relation to the Disease. Dysentery bacilli have been found in large numbers in the dejecta, especially in the early stage of the illness, where they may be present in almost pure culture. They do not, as a rule, spread deeply or tend to invade the general circulation, though some cases of bacteriæmia have been recorded. In the later stages they may be relatively scanty in the stools. Apparently the organisms become progressively less numerous during the illness, and as they diminish, certain other types of intestinal bacteria appear in considerable numbers in the stool, e.g. Morgan's bacillus and allied organisms, paracolon bacilli (*vide* Table, p. 362), *B. fecalis alkali-genes*, *B. proteus*, enterococcus, etc. When complete recovery occurs, these give place to the usual coliform flora. MacConkey's neutral-red-lactose medium has in the past been extensively used for isolation from stools, but recently desoxycholate-citrate agar (also containing neutral-red and lactose) has replaced the former in virtue of its capacity to inhibit coliform bacilli while allowing the dysentery organisms to grow freely. As the typical forms of *B. dysenteriae* are non-lactose-fermenters, the colourless colonies which develop are selected for investigation.

In the severe and acute cases where death may occur in from one to six days, the chief changes are a marked swelling and corrugation of the mucous membrane of the colon, with hæmorrhage and pseudo-membrane at places

There is extensive coagulation-necrosis with fibrinous exudation and abundance of polymorphonuclear leucocytes, and the structure of the mucous membrane, as well as that of the *muscularis mucosæ*, is often lost in the exudation. Sometimes ulceration occurs; and there is also great thickening of the submucosa, with infiltration of leucocytes, these being chiefly plasma cells. In the more chronic forms the changes correspond, but are more of a proliferative character. The mucous membrane is granular, and superficial areas are devoid of epithelium, while ulceration and pseudo-membrane are present in varying degree. In the stools the presence of a large number of markedly degenerate polymorphonuclear leucocytes (along with macrophages and red corpuscles), in the absence of *Entamæba histolytica*, points to bacillary dysentery; and in this way a tentative diagnosis may be made by simple microscopic examination of the stool pending the fuller bacteriological investigation of the case.

Sources and Modes of Infection. Bacillary dysentery is similar to enteric fever as regards its mode of spread. The source of infection is the intestinal excreta of active cases of dysentery and of apparently healthy carriers of the organisms. Carriers include both convalescents and also persons who present no history of a dysenteric or diarrhoeal illness, though, of course, the latter type of carrier is less frequent than the former. In examining carriers it must be remembered that the excretion of the organisms may be intermittent. Dysentery bacilli may be distributed by contaminated water or foods and the house-fly, and also by personal contact and mediate contact. Spread of the disease is the product of insanitary conditions.

Pathogenic Properties. Generally it is impossible to produce any effect on laboratory animals by infection *per os*. Shiga, however, obtained character of young cats. Such attempts. The organism has been previously exalted by intraperitoneal passage. The dysentery bacilli if recently isolated exhibit marked pathogenic effects when introduced intravenously in rabbits, a minute amount of culture being sufficient to produce a lethal result. The organism and its toxic products show marked enterotropism, and inflammatory changes occur in the mucosa of the small and large intestine, frequently with extensive hæmorrhages. The bacilli can be recovered from the contents of the intestine, where they may be present in large numbers. These results may also be produced by subcutaneous injection.

The bacillus produces a toxin which can be separated from fluid cultures by filtration. While this toxin resembles the exotoxins in its specialised action and its ability to incite the formation of a specific antitoxin, its presence in such filtrates is due to autolysis of the bacterial cells following their death in the culture (Okell and Blake). The results of most observers show that the Flexner strains do not produce a similar toxin. In this connection it is noteworthy that in general the Shiga strains, as contrasted with the other varieties, are associated with the most severe clinical form of the disease. The toxin of the Shiga type is extremely active in animals, especially rabbits, and, however introduced into the body, may produce a hæmorrhagic enteritis with often a pseudo-membranous exudate on the surface of the mucosa. The toxin in rabbits and mice acts also on the nervous system with production of muscular paralysis. According to Kanai the effect is on the medulla and spinal cord.

According to Boivin *et al.*, two separate toxins can be distinguished, which differ both in chemical constitution and biological action—(a) a thermostable glyco-lipoid enterotoxin which acts on the intestine of mice; this represents the endotoxin and O antigen, and is a class of compound present in various Gram-negative bacilli (owing to its low toxicity, however, it is doubtful if it should be considered as a specific enterotoxin—Steabben); (b) a protein neurotoxin, which is thermolabile at 75° C. and causes paralysis in mice; its properties are similar to those of exotoxins. According to Morgan and Partridge the former component also contains a protein, while the lipid (phospholipin) is unessential. The so-called neurotoxin produces enterotoxic as well as neurotoxic action, *e.g.* in the rabbit; accordingly, it may be termed neuro-enterotoxin.

Immunity. Both large and small animals have been immunised against the dysentery bacilli and also against toxic filtrates. In the former case the immunisation has been commenced either with non-lethal doses of living cultures, or with cultures killed by heat. The nature of the immunity is probably complex. When cultures have been used, a bactericidal serum is developed in which immune-bodies and complement (*vide* Chapter III) are concerned. When toxin alone is used for immunisation, an antitoxic serum is produced. According to some results, animals immunised with cultures are immune against the toxin, and vice versa. According to Steabben an antiserum developed by injection into rabbits of a killed vaccine of smooth Shiga cultures protects mice against infection with the living smooth organisms (although not against a single lethal dose of the killed bacilli). Such an antiserum does not protect against inoculation with a living rough strain rich in neuro-enterotoxin but lacking smooth antigen, whereas an antiserum developed by a vaccine of the rough strain is an effective prophylactic in this case. The results indicate that an antiserum to be of value for therapeutic as well as prophylactic purposes in man should contain both antitoxic and antibacterial antibodies.

Antisera prepared by immunising large animals against the dysentery bacilli have been extensively applied in the treatment of acute cases. Polyvalent sera have generally been used. Shiga originally reported favourably regarding the therapeutic value of a polyvalent serum used in Japan in large numbers of cases. The active principles of such sera were both antitoxic and antibacterial. While reports as to their practical value have varied considerably, it may be said that in the severe cases, particularly those due to the Shiga type, if sufficiently large doses are given (*e.g.* 200,000 units, as defined under the Therapeutic Substances Regulations) and repeated if necessary, favourable results are obtained. In the most acute cases the serum should be given intravenously.

Prophylactic vaccination. A difficulty attending the application of vaccines is the extremely toxic effect exerted by killed cultures when injected subcutaneously even in relatively small doses. Shiga obviated this by using a sensitised vaccine and this method was followed by others. Various other methods have recently been adopted for modifying toxicity including the use of purified glyco-lipoid antigen and formol-toxoid. Oral administration of dysentery vaccines according to Besredka's method has also been applied and successful results claimed. A further difficulty in the application of vaccines is, of course, the multiplicity of serological types in the dysentery group. Vaccine prophylaxis has not been so extensively applied as to allow of conclusions being drawn regarding its practical value.

BACTERIOPHAGE THERAPY. D'Herelle has advocated the use of a

dysentery bacteriophage in the treatment of the disease, and others have reported favourably on the results of this form of therapy. Its value, however, is doubtful (see Fletcher and Kanagarayer; Riding, Boyd and Portnoy).

OTHER TYPES OF DYSENTERY BACILLI

During the war of 1914-18, bacillary dysentery was extensively investigated, and while the classical types of bacilli formed a large proportion of the strains isolated from cases, 'atypical' varieties were constantly met with. These were a prominent feature of dysentery in the Near East and were found in large numbers in the stools in early cases, the classical types being absent. All such varieties presented characters in common with the recognised dysentery bacilli—being non-motile, Gram-negative bacilli, non-gelatin-liquefying, non-gas-producing, fermenting glucose, but varying in the fermentation of saccharose, mannitol, dulcitol, and lactose, and the production of indole. The dysentery bacilli now designated 'Boyd India' types (*vide supra*), Schmitz type, *B. alkalescens*, *B. dispar*, and 'para-Shiga type' (*vide infra*) correspond to certain biological types reported by Mackie in Egypt during the war of 1914-18. The various types were proved when recently isolated to be extremely virulent on intravenous injection of rabbits, producing a characteristic hæmorrhagic enteritis similar to that resulting from inoculation with the classical strains. As regards the illness due to these organisms, the majority of cases were of the milder types, though severe conditions were not infrequently met with in which 'atypical' organisms were present in large numbers in the early stage.

In recent years further attention has been paid to certain types of dysentery bacilli which differ from the classical forms and some of these have been found responsible for a proportion of cases of dysentery in various parts of the world.

Sonne Type (*Shigella sonnei*). A type of organism biologically and serologically distinct from the Flexner type was recognised by Thjøtta and Sonne as a dysentery-producing organism in Norway, Sweden, and Denmark. This organism is similar to Flexner strains and ferments mannitol, but late fermentation of saccharose and lactose occurs. Indole is not formed. Slow acid formation with clotting may occur in milk. Some strains ferment xylose

Salmonella group. Change from S to R occurs with great rapidity in cultures, thus, on MacConkey's medium, colonies with an otherwise smooth margin often show a projection at one part—'bursting bomb' appearance. It should be noted that colonies when they first appear on MacConkey's medium are colourless though they may redden later. In some cases red papillæ develop from the colonies, analogous to those of *B. coli mutabilis* (p. 320). Cases and outbreaks due to this type have been described in various countries. It has been pointed out that the Sonne type is more resistant to adverse external influences than the classical types, surviving longer in excreta and in water. Strains form a homogeneous type and can be identified by their serological reactions with a specific antiserum. Two major antigenic components have been identified, some strains lack one of these.

Bacillus dispar (*Shigella ceylonensis*) differs from the Flexner type in its fermentation of lactose and saccharose, these sugars often being fermented slowly. It occasionally ferments dulcitol. Indole is formed, and milk is slowly acidified and clotted. It can be differentiated from the Sonne

type by its positive methyl-red reaction and fermentation of sorbitol. Strains are serologically heterogeneous. Some observers have questioned the pathogenicity of this organism. It seems probable, however, that *B. dispar* is etiologically associated with dysenteric conditions which are generally less severe than those due to the typical dysentery bacilli.

Bacillus alkalescens (*Shigella alkalescens*). This organism resembles the Flexner type, but differs in fermenting dulcitol (after one to five days) and in the marked alkalinity it produces in milk. Xylose, rhamnose, and glycerol are also fermented and some strains have been described as fermenting saccharose. Indole is produced. The colonies have an irregular margin, a raised centre, and radial markings. Strains are serologically not completely homogeneous and, though differentiated from Shiga and Flexner strains by agglutination reactions, they share antigenic components with these types (see Nabarro and Edward). This organism shows the same pathogenicity to animals under experimental conditions as the Flexner type; but there has been some controversy whether *B. alkalescens* is a true dysentery-producing organism. In general the evidence indicates that it should be
 thy that it has also been found
 s apart from the intestine.

Bacillus (*Shigella*). This type is now well recognised as a dysentery-producing organism, though it is relatively infrequent in cases and outbreaks as compared with the classical types and the Sonne type. It presents in cultural and biochemical reactions a similarity to the Shiga type, but clearly differs from the latter in its production of indole and in serological characters, though an antiserum for the Shiga type may give some degree of agglutination with the Schmitz type and vice versa. Serologically the Schmitz type is homogeneous; two antigenic components have been identified, but one of these may be lost on continued laboratory cultivation of the organism. Strains vary in their toxicity and though some are highly toxic, they do not equal the toxicity of the Shiga type. As in the case of the Shiga organism, a thermolabile neurotoxin is produced by some strains (Buchwald).

Newcastle Type. This organism was originally reported in outbreaks of dysentery by Clayton and Warren and is now recognised as a common member of the dysentery group. It has the general characters of the group and when grown in the ordinary sugar media may appear to be non-gas-producing. It was originally pointed out, however, that if Lemco broth is used as a basis for the media gas production occurs; and in this respect the organism would present a distinct difference from other members of the group. It seems likely that the character of the peptone used is also a factor in determining gas production (Ewing and Taylor). Gas production, however, may often not amount to more than a 'bubble' in a Durham's tube. Glucose and maltose are fermented and usually dulcitol, but not lactose, saccharose, or, as a rule, mannitol. (Strains presenting similar characters to the Newcastle type but differing in the fermentation of mannitol have sometimes been described as the 'Manchester type.') Indole is not produced. Serologically the Newcastle type is homogeneous and is similar to the Flexner VI subtype (corresponding to Boyd's strain '88').

Para-Shiga Type. This designation has been given to strains resembling in bioch Such
 type, b quhart;
 strains considered
 they have been reported in recent years by various workers and considered
 to be dysentery-producing organisms. A large number of strains isolated in

India and Egypt have been described by Sachs and classified into eight serological subtypes. He has also arranged them in four groups according to the fermentation of arabinose and the production of indole. Two of the groups are identical in biochemical reactions with the Shiga and Schmitz types respectively, but are not agglutinated by antisera for these organisms.

ORGANISMS ASSOCIATED WITH INFANTILE DIARRHŒA, AND MORGAN'S BACILLUS

The etiology of this condition, so prevalent in infants and children in the warmer seasons of the year, has attracted considerable attention from the bacteriological standpoint. The condition, however, cannot be regarded as due to a single specific agent. Certain of the more severe cases occurring in this country and in other temperate climates are found on investigation to be due to the dysentery bacilli, mostly of the Flexner or Sonne type, though the

application of the term. Some cases and outbreaks are due to members of the *Salmonella* group.

An investigation of the disease in Britain was made in 1906 by Morgan, who found no evidence of the association of dysentery bacilli with the condition. In 63 per cent of the cases investigated, however, he isolated from the stools and intestine a type of organism now generally designated 'Morgan's No. 1 bacillus' (*Salmonella* or *Proteus morgani*), which is a motile Gram-negative bacillus with the general characters of the coli-typhoid group and possessing characteristic fermentative reactions. It produces acid and usually slight gas in glucose, but has no effect on lactose, saccharose, mannitol, maltose, or dulcitol; it produces indole, gelatin is not liquefied. Growing at 37° C. Morgan's bacillus produces discrete colonies, but at 20° to 28° C. on 1 per cent agar it yields a spreading type of growth like that of *B. proteus*. Strains are serologically heterogeneous, with group-specific H antigens and type-specific O antigens. A relationship with *B. proteus* through its H antigenic structure has been demonstrated. Some strains produce a hæmolyisin for sheep's blood. Some are stated to yield late fermentation of saccharose. Though Morgan's bacillus does not liquefy gelatin, in view of its other characters systematists have, recently classified it with the *Proteus* group. As mentioned, it shows relationships also with the coli-typhoid group. Morgan's bacillus causes diarrhœa and death in young rabbits, rats, and monkeys, when these animals are fed on cultures, and produces also a fatal infection on intraperitoneal injection of mice.

Morgan also found that in diarrhœa cases, the lactose-fermenters, so characteristic of normal fæces, are relatively less numerous and tend to be replaced by non-lactose-fermenting types. In simple diarrhœal conditions, apart from clinical dysentery, various non-lactose-fermenting Gram-negative bacilli, e.g. Morgan's bacillus and similar types, paracolon bacilli, *B. faecalis alkaligenes*, *B. proteus*, etc., may be met with in stools and are present often in very large numbers, almost replacing the usual coliform bacilli. These organisms are also met with as concomitants of the dysentery

trons capable of replacing the usual coliform flora. The evidence collected on

this subject is highly suggestive, however, that certain of these organisms are pathogenic entities responsible for diarrhoeal conditions not only in young subjects, but also in adults.

The further question arises whether in infants, and particularly during the neo-natal stage when natural resistance is little developed, organisms which form the normal flora of the intestine may act as causal agents of enteritis and diarrhoea, *e.g.* the ordinary coliform bacilli, faecal streptococci, and anaerobes such as *B. welchii*. In this connection it is noteworthy that coliform bacilli may be actively pathogenic in calves, as in the condition of 'white-scours', and one type of *B. welchii* is the causal organism of lamb dysentery. It has been long recognised that diarrhoeal conditions are more frequent in artificially fed infants in whose intestine coliform organisms are abundant, than in breast-fed infants in whom such organisms are relatively less numerous. It has also been suggested that certain types of coliform bacilli possessing a high degree of virulence may be responsible for outbreaks of diarrhoea. The occurrence of such outbreaks in institutions would, of course, point to a specific pathogenic agent spread from person to person or derived from a common source. It is certainly significant that on bacteriological examination of many cases of infantile diarrhoea no unusual organisms can be detected in the bowel or dejecta and the possibility that a virus is the responsible agent requires consideration, particularly when such cases occur in epidemic form. In some outbreaks of enteritis of the type described above adults as well as young subjects have been affected and signs of faucial infection have been noted; this also might suggest a virus origin (*vide* p 598); but at present no consistent evidence of the virus etiology of infantile diarrhoea is available. (See Bradley; Reimann, Stuart, Christensen, Hargreaves).

BACTERIOLOGICAL DIAGNOSIS OF BACILLARY DYSENTERY

This consists essentially in the isolation of the dysentery bacillus from the intestinal dejecta and its identification by the methods, and according to the criteria, described above.

The stool should be as fresh as possible since when kept under ordinary conditions dysentery bacilli may not survive for more than a few hours and are overgrown by other intestinal organisms. The collection of specimens is referred to in the Appendix. If there is likely to be delay in the examination of the specimen, it should be mixed with the glycerol-saline solution described under the diagnosis of enteric fever. In some cases a suitable specimen for diagnosis can be obtained by rectal swab and this is particularly useful in examining children.

It is valuable in the first place to examine the specimen microscopically by taking a loopful of any mucus present and emulsifying it in a drop of saline solution on a slide, the preparation being then covered with a coverslip. In bacillary dysentery there is usually a copious cellular exudate consisting of degenerate polymorph leucocytes, and macrophage cells. In a case of clinical dysentery of unknown cause, this cellular exudate (with the absence of amœbæ) points to a bacillary infection. (Of course the dysentery bacilli cannot be recognised microscopically, being similar in appearance to the coliform organisms normally present.)

Cultures are then made from the specimen on a plate of desoxycholate-citrate agar, as in the examination of stools from cases of enteric fever. (Any piece of mucus present in the specimen should be rinsed in saline and then rubbed well on the medium.) The use of this medium which

is inhibitory to coliform bacilli, allows a heavy inoculation to be made on the plate (In the past MacConkey's medium has been much used for the same purpose, but has now been largely replaced by desoxycholate-citrate agar.) The colonies of dysentery bacilli can be recognised by their colourless or pale appearance as compared with the red-coloured coliform colonies, but of course other non-lactose-fermenters may also present pale colonies, it is therefore necessary to subculture at least three of the suspected colonies, including different types of such colonies if they vary in their naked-eye appearances. Certain dysentery bacilli are lactose-fermenters, but this may be a slow reaction, *e.g.* Sonne type, and the colonies seen on desoxycholate or MacConkey's medium after twenty-four hours' incubation are still colourless.

The colony subcultures after growth are examined for the various characters of the dysentery bacilli as described earlier in this chapter, particular attention being paid to the absence of motility, biochemical reactions (in glucose, lactose, dulcitol, saccharose, and mannitol) and the production of indole, and according to these the type can generally be identified in a preliminary way, but this requires to be confirmed by agglutination reactions with specific antisera, *e.g.* for the Shiga type, Flexner type (a polyvalent antiserum being used), Sonne type, and Schmitz type. If it is considered neces-

biochemical tests referred to above also serve for the identification of other non-lactose-fermenting organisms which may be met with in dysenteric or diarrhoeal cases, *e.g.* Morgan's bacillus, paracolon bacilli, etc. At post-mortem examination the base of a suspicious intestinal ulcer is scraped down to the muscle and the scrapings used to inoculate plates of desoxycholate-citrate medium.

delayed or completely absent during the illness. Agglutination of the Shiga type in a titre of 1 in 50 is usually accepted as of diagnostic significance. The Flexner type is more susceptible to normal serum and results with lower dilutions than 1 in 100 cannot be taken as indicating the presence of infection. The serological heterogeneity of the Flexner type involves testing with each subtype. Agglutination of the Sonne type even in a dilution of 1 in 20 is regarded as significant. The multiplicity of serological types, the weakness of the reaction, and in many cases the absence of a specific reaction renders the serological method of relatively little practical value, and in all cases the isolation of the infecting organism constitutes the most conclusive means of diagnosis.

BACILLUS PYOSEPTICUS EQUI (SHIGELI 1 VISCOSA)

This organism has been classified by some systematists in the genus *Shigella*, but probably its true affinities are with the pneumobacillus. It has been found in equine purulent nephritis and in 'joint ill' of foals and has also been reported in pyæmia of pigs. It is a Gram-negative non-motile pleomorphic bacillus, which in lesions may be capsulate and occurs in masses or threads. It grows aerobically on ordinary media and the colonies are often of a mucoid type while in broth it yields a slimy deposit. It ferments glucose, lactose, saccharose, and mannitol with acid but no gas production. It does not produce indole and does not liquefy gelatin. The methyl red and Voges-Proskauer reactions are negative and citrate is not utilised. Pathogenicity has not been demonstrated in laboratory animals.

TABLE SHOWING CHARACTERS OF REPRESENTATIVE ORGANISMS OF THE COLI-TYPHOID GROUP

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	Motility	Gelatin	Glucose	Lactose	Dulcitol	Saccharose	Mannitol	Adonitol	Inulin	U Inositol	X Xylose	Arabinose	Rhamnose	Milk	Indole	Voges-Proskauer	Production of H ₂ S	Methyl-Red reaction	Utilisation of Sodium citrate	Utilisation of Sodium tartrate
<i>B. coli</i> communis (Escherich)	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. aerogenes</i> type	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>Pneumobacillus</i> type	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. coli</i> <i>anaerogenes</i> type	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
Morgan's bacillus and related organisms	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. typhosus</i>	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. paratyphosus</i> A	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. paratyphosus</i> B	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. enteritidis</i> (Gaertner)	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. aertrycke</i>	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. suis</i> <i>pasteur</i>	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. paratyphosus</i> C (Hirschfeld)	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. pullorum</i>	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. gallinarum</i>	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. dysenteriae</i> Shiga type	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. dysenteriae</i> Flexner type	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+
<i>B. dysenteriae</i> Sonne type	+	-	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	AG	+	+	+	+	+	+

+ In Motility column = presence of motility. In Gelatin = liquefaction. In Indole = formation of indole. In Voges-Proskauer = presence of reaction in other column = presence of reaction.
 - In Motility column = absence of motility. In Gelatin = no liquefaction. In Indole = absence of indole formation. In Voges-Proskauer = absence of reaction in other column = absence of reaction.
 AG = Acid production; C = Gas; A = Alk. = development of alkali.
 Note:—In general only those characters are given which are important in identification and differentiation.

¹ Strains from gastro-enteritis may be +

² Monophasic type (Kunzendorf) produces H_2S

³ From *Maltose* acid (but no gas) may be formed or there may be no change

⁴ Tested by growing in nutrient agar containing 0.01 per cent basic lead acetate, blackening of the medium indicates H_2S production

⁵ Tested in Hoser's medium

Biochemical reactions of certain *Salmonella* types not included in the table may be broadly summarised as follows by comparison with *B. enteritidis*

- (1) Similar: *Aberdeen*, *anatum*, *Dublin* (but fermentation of xylose, arabinose, and rhamnose is variable)
Newport, *Oranienburg*, *Senftenberg*, *Stanley* (*Senftenberg* var. *Newcastle* does not produce H_2S)
- (2) Similar, but some strains do not ferment dulcitol, xylose, arabinose, or rhamnose, and H_2S production is variable: *abortus* *ovis*
- (3) Similar, but some strains ferment inositol: *Chester*, *Eastbourne*, *Montevideo*, *Reading* (fermentation of

horse serum agar (Bhatnagar). - The bacilli do not form spores, and are non-motile. They stain readily with the basic aniline stains, and are Gram-negative.

CULTURAL CHARACTERS. From the affected glands, etc., the bacillus can readily be cultivated aerobically on the ordinary media. It is also a facultative anaerobe. It grows well at the temperature of the body, though the optimum temperature of freshly isolated strains is lower, about 27° C., and growth occurs even at 14° C. In general the organism grows rather slowly in primary cultures. Attention has been drawn to the sensitiveness of *B. pestis* to atmospheric oxygen and the difficulty of initiating growth when only a small number of organisms are inoculated on plates of culture medium; this difficulty is obviated if blood or sodium sulphite (0.025 per cent) is added or if air is excluded (Schütze and Hassanein). Wright has pointed out that the organism may be killed by exposure to more than 1 per cent. of oxygen at a temperature of 37° C. On *agar* and on *serum media* the colonies are whitish circular disks of somewhat transparent appearance, and with a smooth, shining surface. When examined with a lens, their borders appear slightly wavy. After several days' incubation the colonies become larger, reaching 3 to 4 mm. in diameter, and develop a raised opaque centre with a thin transparent border. Growths sometimes have a viscous consistence. When *agar* cultures are kept at room temperature some of the colonies may show a luxuriant growth which is more opaque than the rest of the culture, the appearance, in fact, being often such as to suggest the presence of a mixed growth. Individual colonies may thus vary considerably in character, but such variations do not as a rule constitute stable differences. In stab cultures in *gelatin*, growth takes place along the wire track as a white line, composed of small spherical colonies. On the surface of the *gelatin* a thin, semi-transparent layer may form, which is usually restricted to the region of puncture, though sometimes it may spread to the wall of the tube. There is no liquefaction of the medium. In *broth* the growth usually forms a slightly granular or powdery deposit at the foot and sides of the container, somewhat resembling that of a streptococcus. If oil or melted butter is added to the broth so that drops float on the surface, then a striking form of growth may result, to which the term 'stalactite' has been applied. This consists in the growth starting from the under surface of the fat globules and extending downwards in the form of pendulous, string-like masses. These masses are exceedingly delicate, and readily break off on the slightest shaking; accordingly during their formation the culture must be kept absolutely at rest. This manner of growth constitutes an important but not specific character of the organism, it is not exhibited by all strains of the organism, and varies from time to time with the same strain.

Smooth and rough types of colonies have been differentiated, but it is doubtful if these differences constitute a true S→R variation as in other bacterial groups.

BIOCHEMICAL REACTIONS. The plague bacillus has only weak fermentative properties and gas is never produced; with a number of fermentable substances varying results have been recorded. Of practical importance in differentiating *B. pestis* are the constant findings that it forms acid from glucose, but not from lactose or saccharose. Other substances from which acid is formed are *lævulose*, mannitol, arabinose, maltose (usually), xylose (sometimes), rhamnose (sometimes); whereas acid is not produced from raffinose, sorbitol, or dulcitol (usually). It does not form indole. The methyl-red reaction is positive. Growth is not inhibited on a bile-salt medium. Nitrite is produced, and methylene blue is not reduced (Topley

and Wilson). Hydrogen sulphide is not formed or only very slightly. The organism has no hæmolytic properties.

VIABILITY. The organism in its powers of resistance corresponds with other spore-free bacilli, and is readily killed by heat, an exposure for fifteen

drying have given somewhat diverse results, but as a rule the organism has been found to be dead after being dried in air for one or two days, though

the animal body, but in dried flea faeces (*vide infra*) it has been found to persist for as long as five weeks.

Toxin. As is the case with most organisms which extensively invade the tissues, the toxin in plague cultures is chiefly contained in the bodies of the bacteria. Injection of dead cultures in animals produces distinct toxic effects, *post mortem*, hæmorrhage in the mucous membrane of the stomach, and areas of necrosis in the liver and at the site of inoculation, may be present. The toxic substance is comparatively resistant to heat, being unaffected by exposure to 65° C for an hour.

Pathological Effects and Distribution of Bacilli. The disease occurs in different forms, the *bubonic* and the *pulmonary* being the best recognised, to these may be added the *septicæmic*. The most striking feature in the *bubonic* form is the affection of the lymphatic glands, which undergo intense inflammatory swelling, attended with hæmorrhage, and generally ending in a greater or less degree of necrotic softening if the patient lives long enough. The connective tissue around the glands is similarly affected. The bubo is thus usually formed by a collection of enlarged glands fused by the inflammatory swelling. True suppuration is rare. Usually one group of glands is affected first, constituting the primary bubo—in the great majority the inguinal or the axillary glands—and afterwards other groups may become involved, though to a much less extent. Along with these changes there is

large numbers in the swollen glands, being often so numerous that a film

disorganisation of the gland has occurred, they become irregularly mixed with the cellular elements. Later still they gradually disappear, and when necrosis is well advanced it may be impossible to find any—a point of importance in connection with diagnosis. In the spleen they may be very numerous or they may be scanty, according to the amount of blood infection which has occurred, in the secondary lesions mentioned they are often abundant. In the *pulmonary* form the lesion is the well-recognised 'plague pneumonia'. This is of broncho-pneumonic type, though large areas may be formed by confluence of the consolidated patches, and the inflammatory process is usually attended by much hæmorrhage, the bronchial glands show inflammatory swelling. Clinically there is usually a fairly abundant frothy sputum often tinted with blood, and in it the bacilli may be found in large numbers.

Sometimes, however, cough and expectoration may be absent. The disease in this form is almost invariably fatal; it is also extremely infectious. In the *septicæmic* form proper there is no primary bubo discoverable, though there is almost always slight general enlargement of lymphatic glands; here also the disease is of specially grave character. A bubonic case may, however, terminate with septicæmia, in fact, all intermediate forms occur. In the various forms of the disease the bacilli occur also in the blood, in which they may be occasionally found during life by microscopic examination, chiefly, however, just before death in very severe and rapidly fatal cases. The examination of the blood by means of blood smears is a very reliable procedure. It may be that the bacillus may be isolated



FIG. 85. Septicæmic form of plague. (Blood smear.)

number will necessarily vary in different epidemics. The Advisory Committee appointed by the Secretary of State for India in 1905, found that in some septicæmic cases the bacilli may be present in the blood in large numbers two, or even three, days before death, though this is exceptional.

The above types of the disease are usually classified together under the heading *pestis major*, but there also occur milder forms to which the term *pestis minor* is applied. In the latter there may be a moderate degree of swelling of a group of glands, attended with some pyrexia and general malaise, or there may be little more than slight discomfort. Between such and the graver types, cases of all degrees of severity are met with. In recovered cases small numbers of the bacilli may remain alive and virulent in lymph glands for many months.

Experimental Inoculation. Guinea-pigs, mice, rats, rabbits, and various other rodents are susceptible to inoculation, the first being on the whole most suitable for experimental purposes. After subcutaneous injection there



* 11/11/11 11:11

established by the work of the Advisory Committee referred to above. It had previously been shown that when fleas were allowed to feed on animals suffering from plague, plague bacilli might be found for some time afterwards in the stomach, and some observers, for example Simond, had succeeded in transmitting the disease to other animals by means of the infected insects. Most observers, however, had obtained negative results, but the Committee showed by carefully planned experiments that the disease could be transmitted from a plague rat to a healthy rat, kept in adjacent cages, when fleas were present; whereas this did not occur when means were taken to prevent the access of fleas, though the facilities for aerial infection were the same. The disease could also be produced by fleas removed from plague rats and transferred directly to healthy animals, success having been obtained in fully 50 per cent. of experiments of this kind. When plague-infected guinea-pigs were placed among healthy guinea-pigs, comparatively few of the latter acquired the disease if fleas were absent or scanty; whereas all of them might die of plague when fleas were numerous. This result demonstrated the comparatively small part played by direct contact, even when of a close character. Important results were also obtained with regard to the mode of infection in houses where there had been cases of plague. It was found possible to produce the disease in susceptible animals by means of fleas taken from rats in these houses. When animals were placed in plague houses and efficiently protected from fleas they remained healthy; whereas they acquired the disease when the cages were free to the access of fleas in the neighbourhood.

The following are some of the experiments which were conducted. A series of six huts were built which only differed in the structure of their roofs. In two the roofs were made of ordinary native tiles in which rats freely lodge; in two others, flat tiles were used in which rats live, but in which they have not such facilities for movement as in the first set, and in the third pair the roof was formed of corrugated iron. Under the roof in each case was placed a wire diaphragm which prevented rats or their droppings having access to the hut, but which would not prevent fleas falling down on to the floor of the hut. The huts were left a sufficient time to become infested with rats, and then on the floor in each case healthy guinea-pigs mixed with guinea-pigs artificially infected with plague were allowed to run about together. In the first two sets of huts to which fleas had access the healthy guinea-pigs contracted plague, while in the third set they remained unaffected, though they were freely liable to contamination by contact with the bodies and excreta of the diseased animals. But when accidentally fleas obtained admission to the third set of huts then infection of the un inoculated animals commenced. Other experiments were also performed. In one case healthy guinea-pigs were suspended in a cage where animals were running. In another case the cage were suspended in a cage where infection took place. In a third case the cage which contained infected animals was surrounded by a zone of sticky material broader than the jump of a flea, another was left without this protection. The monkey in the former cage remained unaffected, but the other monkey contracted plague.

In all these experiments the common rat-flea of India—*Xenopsylla cheopis*—was the vector, but it was proved that this flea also infests and bites the human subject. In addition to *X. cheopis* certain other species of rat-fleas may also transmit the infection to man, e.g. *Ceratophyllus fasciatus*. All observations show that not only is plague transferable by means of fleas, but that this is practically the only method obtaining in natural conditions, with the exception that rats may become infected by eating the carcases of other animals containing large numbers of plague bacilli. It is improbable from the experiments made that bubonic plague is transmitted by direct contact even when of a close nature, in fact, it has been shown that plague-infected guinea-pigs may suckle their young without

the latter acquiring the disease. Other experiments showed that when plague bacilli were placed on the floors of houses, they died in a comparatively short period of time. After forty-eight hours it was not found possible to reproduce plague by inoculation with material from floors which had been grossly contaminated with cultures of the bacillus.

The value with regard to this some further facts may be given. Plague in Bombay occurs in two chief species of rats, the *Rattus rattus*, the black house-rat, and *Rattus norvegicus* (*decumanus*), the common brown rat of the sewers. The former, owing to its presence in dwelling-houses, is chiefly responsible for the transmission of the disease to man, while the latter, on account of the large number of fleas which infest it, is of special importance in maintaining the disease from season to season. The year may be divided into two portions—an epizootic season, from December to May inclusive, and a non-epizootic, from June to November. During the latter period there are few cases of plague in rats on account of fleas being scanty, especially is this so in the case of *Rattus rattus*. In fact, in certain villages where this species alone is present, the disease may actually die out at the end of the epizootic season, and accordingly when plague reappears in these places this is due to a fresh importation—a fact of great practical importance. A fresh epizootic first affects chiefly *Rattus norvegicus*, and a little later spreads to *Rattus rattus*, while later still the disease attacks the human subject in the epidemic form, in each case fleas are the vectors of transmission, and an interval of from ten to fourteen days intervenes between the outbreak of the epizootic and that of the epidemic. The proportion of cases of plague in *Rattus norvegicus* is much higher than in *Rattus rattus*, for the reason mentioned. It has been shown that a large proportion of the fleas removed from plague-infected rats contain plague bacilli, and that the fleas may remain infective for a considerable number of days, sometimes for a fortnight.

After a flea has sucked infected blood from an animal the bacilli multiply in the stomach and proventriculus, so that the latter structure becomes more or less blocked by the growth of bacilli. When this insect bites a new host the suction of blood, owing to the blockage, is followed by regurgitation of blood into the bite puncture along with bacilli which have been growing in the œsophagus and proventriculus or the stomach. In this way a heavy inoculation results (Bacot and Martin).

disease to animals by means of fleas is more frequently successful at lower temperatures, and it has been shown that a temperature about 50° F. and an atmospheric humidity approaching saturation constitute the most favourable conditions for the survival of the organisms in the insect. Though plague bacilli may survive in fleas for some weeks the infectivity of the insect is usually limited to seven to fourteen days.

As regards the dying out of epidemics, some interesting facts were brought forward by Liston. He and his co-workers showed that rats taken from different towns vary greatly in their susceptibility to inoculation with plague

regard to their young. The diminution of plague amongst rats, and thus the subsidence of an epidemic, accordingly depends on the killing-off of the more susceptible animals.

Enzootic and epizootic plague may occur in other wild rodent animals, e.g. ground-squirrels, wood rats, and white-tailed prairie 'dogs' in the western United States, gerbilles and multimammate mice in South Africa, the tarabagan or Siberian marmot in Manchuria, spermophiles and field-mice in south-west Russia; in these animals transmission takes place by various species of fleas and possibly in some cases by lice and ticks. The infection in such wild rodents sometimes gives rise to human cases or outbreaks—the so-called 'sylvatic plague'.

Plague pneumonia appears to occur first of all as a complication in a bubonic case, and there is no evidence that the bacilli differ in virulence in the two conditions. In epidemic pneumonic plague, from a consideration of the anatomical changes and the clinical facts, the disease may be said to be spread from person to person and to be produced by the direct passage of the bacilli into the respiratory passages by inhalation. The secretion droplets sprayed into the air by infected persons in the act of coughing carry the plague bacilli. Accordingly a case of plague pneumonia may be of great infectivity in producing other cases of plague pneumonia. It has been shown that droplet infection is effective within a range of three feet between persons (Wu Lien Teh). Pneumonic plague prevails in the winter season when the weather is cold and wet with resulting overcrowding in ill-ventilated insanitary dwellings. It has also been found that plague bacilli die quickly in a dry atmosphere, whereas they survive best under conditions of cold and high humidity (Teague and Barber). Small epidemics of plague pneumonia break out from time to time, for example, in 1911 an extensive epidemic occurred in Manchuria leading to 50,000 deaths in six months. In this epidemic, direct infection from patient to patient was clearly shown. It may be noted here that accidental pneumonic plague has occurred among laboratory workers through inhalation of infective material or cultures (see Petrie).

Immunity. By the injection of dead cultures in suitable doses, a certain degree of immunity against the living virulent bacilli is obtained, and, as first shown by Yersin, Calmette and Borrel, the serum of such immunised animals confers a degree of protection on small animals such as mice. On these facts the principles of preventive inoculation and serum treatment, presently to be described, depend. It may also be mentioned that the filtrate of a plague culture possesses a very slight toxic effect. The whole blood of animals immune to *B. pestis* contains a much greater number of the organisms *in vitro* than the blood of normal animals. This increased activity is due to specific antibodies in the plasma (or serum). Also, in immune animals the bacilli when injected into the subcutaneous tissue are fixed to a great extent at the site of inoculation, in a manner not shown by susceptible animals. This fixation reaction appears to be independent of lymphatic blockage or deposition of fibrin (see Jawetz and Meyer).

Preventive vaccination. Haffkine's method of preparing plague vaccine has been extensively used. Cultures are made in flasks of broth with drops of oil on the surface (in India Haffkine employed a medium prepared by digesting goat's flesh with caustic soda). The flasks are shaken every few days so as to break up the staccato fresh crops. The flasks are kept at a temperature of about 25° C., and

growth is allowed to proceed for about six weeks. Sterilisation has usually been effected by exposing the contents of the flasks to 55° C. for a quarter of an hour (*vide infra*) ; thereafter phenol is added in the proportion of 0.5 per cent. The contents are well shaken to diffuse thoroughly the sediment in the fluid, and are then distributed in small sterilised bottles for use. The vaccine thus contains both the dead bodies of the bacilli and any toxins which may be in solution. It is administered by subcutaneous injection usually in a single dose of 4 c c. The method has been systematically tested by inoculating a certain proportion of the inhabitants of districts exposed to infection, leaving others uninoculated, and then observing the proportion of cases of disease and the mortality among the two classes. The results of inoculation have been distinctly satisfactory. For although absolute protection is not afforded by inoculation, both the proportion of cases of plague and the percentage mortality among these cases have been considerably smaller in the inoculated as compared with the uninoculated. Protection is not established till some days after inoculation, and lasts for a considerable number of weeks, possibly for several months (Bannerman). In the Punjab during the season 1902-3 the case incidence among the inoculated was 1.8 per cent, among the uninoculated 7.7 per cent, while the case mortality was 23.9 and 60.1 per cent respectively in the two classes, the statistics being taken from villages where 10 per cent. of the population and upwards had been inoculated. According to statistics more recently collated by Taylor in India, vaccination with Haffkine's prophylactic yields roughly a fourfold protection against attacks and an eightfold protection against death.

Plague vaccines have also been prepared from cultures on solid medium in accordance with the general methods used for vaccine preparation. Immunisation experiments in animals have indicated, however, that the use of living non-virulent cultures as vaccine produces a higher degree of immunity than results from injecting killed organisms. This type of vaccine has been applied with success in the Dutch East Indies (Vogel), but since it loses its properties on keeping, this presents difficulties of distribution, especially in a large country. According to Bhatnagar effective immunisation is produced only by those non-virulent strains which develop well defined capsular material when growing at 37° C. Sokhey and Maurice pointed out the importance of using a fully virulent strain in preparing the Haffkine vaccine. They showed that the original practice of killing the organisms at 60° to 70° C. injures the immunising properties of the vaccine, and an exposure at 55° C. for fifteen minutes is preferable. The final preparation was standardised by protection tests in white mice, and apparently such a heat-killed vaccine from a virulent strain was more effective in these animal tests than a vaccine consisting of a non-virulent living culture, but the latter finding has not been generally confirmed.

Serological reactions. Specific agglutinins may appear in the blood of patients suffering from plague, as also they do in the case of animals immunised against the plague bacillus. It is to be noted, however, that in clinical cases the reaction is not invariably present, the potency of the serum is not of high order, and the carrying out of the test is complicated by the natural tendency of the bacilli to cohere in clumps. The usual macroscopic method is used for the reaction. A suspension of the culture is mixed with the serum, and the mixture is allowed to stand for some time. The reaction is best obtained with the German Plague Commission and the observations of Cairns, made during the Glasgow epidemic, it may be said that the reaction is best obtained with

dilutions of the serum of from 1 in 10 to 1 in 50. Cairns found that the date of its appearance is about a week after the onset of illness, and that it usually increases till about the end of the sixth week, thereafter fading off. It is most marked in severe cases characterised by an early and favourable crisis, less marked in severe cases ultimately proving fatal, while in very mild cases it is feeble or may be absent. The method, if carefully applied, may be of service under certain conditions; but it will be seen that its use as a means of diagnosis is restricted. The use of high-titre agglutinating antisera obtained from horses is of value for identifying cultures of *B. pestis*; there is, however, group agglutinating action also on *B. pseudotuberculosis rodentium* (*vide infra*).

Antigenic structure. It has been shown by Schütze that the plague bacillus possesses two types of antigen. One of these, which is thermolabile at 100° C., is contained in the envelope or the body of the organism, is thermosta to Schütze, is only fully formed when the organism is growing at 37° C., and cultures grown at lower temperatures, e.g. 25° C., as in the Haffkine vaccine, may lack this antigen. Schütze has pointed out that vaccines prepared from cultures grown at the higher temperature possess greater immunising properties in virtue of the envelope antigen which they contain, and his work offers a criticism of the standard method which has been used for preparing the plague vaccine in India. He has also correlated the protective value of anti-plague sera with their content of antibody for the envelope antigen. As mentioned above, this antigen is sensitive to heat and its preservation in a heat-sterilised vaccine requires careful consideration. Heating at 100° C. for fifteen minutes may render the vaccine useless; the antigen is also damaged by heating at 70° C. Thirty minutes' exposure at 56° C. before addition of phenol does not, however, affect the immunising properties of the vaccine. In further studies of the antigens of the plague bacillus, Schütze found that while prophylaxis by a heat-killed vaccine in the rat depends on the envelope antigen, in the mouse this constituent has a less marked effect. In neither animal were vaccines from virulent cultures more potent than avirulent organisms if the cultures were killed, and likewise no difference in potency was apparent between smooth and rough strains. He observed no serological difference between virulent and avirulent strains or between smooth and rough variants. No essential antigenic differences exist among strains of *B. pestis*. The antigenic relationships of *B. pestis* to other members of the *Pasteurella* group will be dealt with later.

Anti-plague sera. Of these, two have been used as therapeutic agents, namely, that of Yersin and that of Lustig. Yersin's serum was prepared by injections of increasing doses of plague bacilli into a horse. In the early stages of immunisation dead bacilli were injected subcutaneously, thereafter into the veins, and, finally, living bacilli were injected intravenously. Of this serum 10 to 20 c.c. have been used, and injections have usually been repeated on subsequent days. Lustig's serum was prepared by injecting a horse with repeated and increasing doses of a substance derived from the bodies of plague bacilli, probably in great part nucleo-protein. Masses of growth were obtained from the surface of agar cultures, and were broken up and dissolved in a 1 per cent solution of caustic potash. The solution was then made slightly acid by hydrochloric acid, when a bulky precipitate formed; this was collected on a filter and dried. For use, a weighed amount was dissolved in a weak solution of carbonate of soda and then injected. Extensive observations with both of these sera show that neither of them can be considered a powerful remedy in cases of plague, though in certain instances favourable results have been recorded. The Indian Commission came to the conclusion 'that, on the whole, a certain amount of advantage accrued to the patients in cases both of those injected with Yersin's serum and of those injected with Lustig's serum'. It may also be mentioned that the Commission found that Yersin's serum modified favourably the course of the experimental disease in animals, whereas Lustig's serum had no such effect.

By immunising cattle with a virulent strain of *B. pestis* a highly potent antiserum, as judged by experimental tests, was obtained by Naidu, F. P. Mackie and Brist. Pirie and Grasset have found that serum produced by immunisation with living avirulent organisms is more potent in animal protection tests than that obtained with dead cultures, and Girard has claimed a fairly high percentage of successful results with such serum in the treatment of human plague.

Bacteriological Diagnosis. Where a bubo is present a little of the 'juice' may be obtained by puncture and aspiration with a sterile hypodermic syringe. The fluid is then examined microscopically, and cultures on agar and blood agar should be made by the successive stroke method. The morphological, cultural, and biochemical characters are investigated. The pathogenic properties should also be studied, the guinea-pig being on the whole most suitable for subcutaneous inoculation. According to Sokhey, however, the white mouse is the most susceptible animal and the most uniform in its behaviour, the subcutaneous injection of even ten bacilli from a virulent

glands. The organism may be obtained in culture from the blood in a considerable proportion of cases by withdrawing a few cubic centimetres and proceeding in the usual manner. On the occurrence of the first suspected case, every care to exclude possibility of doubt should be used before a positive opinion is given.

In a case of suspected plague pneumonia, in addition to microscopic examination of the sputum, the above cultural methods and animal inoculation with the sputum should be carried out. Smearing the nasal mucous membrane or a freshly shaved area of skin of a guinea-pig may be recommended. Here a positive diagnosis should not be attempted by microscopic examination alone, especially in a plague-free district, as bacilli morphologically resembling the plague organism may occur in the sputum in other conditions.

Recognition of Plague Infection in Rats and other Wild Rodents. This is of great importance in plague prevention. In the autopsy of a rat suspected to be the subject of an acute infection with *B. pestis* careful attention is first paid to the various groups of lymph glands, particularly those of the neck which are most frequently affected. The glands show marked enlargement with surrounding oedema and often haemorrhages, and plague bacilli can almost always be demonstrated in smears from the tissue. The liver shows often a characteristic mottling due to yellow necrotic areas, and effusion into the pleura is noted. There is frequently marked congestion of the subcutaneous vessels, often with haemorrhages, and the spleen is congested and enlarged. In an animal recently dead the plague bacilli may be demonstrated in the spleen and heart blood. It often happens, however, that carcases of rats found dead and requiring examination for plague infection are decomposed, and it may be impossible to demonstrate the bacilli microscopically and isolate them by the usual methods of culture. The existence of the infection may sometimes be established in such cases by inoculating a shaved and scarified area of skin in a guinea-pig with material from the lesions. The plague bacilli invade the tissues and produce a typical plague infection. All the characters of a suspicious organism must be investigated since recently isolated strains of coliform bacilli on subcutaneous inoculation in guinea-pigs or rats may cause lesions closely resembling those due to *B. pestis* (Levinthal), and in rats other *Pasteurella* organisms may have a similar action. It should

reported. The associated clinical conditions have been varied, *e.g.* puerperal fever, gastro-enteritis following handling of infected fowls, appendicitis, empyema, meningitis after fracture of the skull, local or blood infection following the bite of various animals (cat, dog, rabbit, panther); in certain of these last-mentioned the condition has been complicated by osteomyelitis or cellulitis with adenitis (see Regamey; Ludlam; Alliot *et al*). The occurrence of such organisms in the upper air passages of certain animals would explain infection following bites, though they are not usually found in dogs. In some of the reported human cases it has not been possible to trace the infection to an animal source.

Bacillus pseudotuberculosis rodentium has also been reported in a few human cases. In a fatal case recorded by Topping *et al.* in California, the condition was an acute febrile disease with supervening jaundice and the liver *post mortem* was found to be studded with small necrotic foci.

CHAPTER XVII

THE ORGANISMS OF UNDULANT FEVER AND EPIZOOTIC ABORTION (BRUCELLA GROUP)

UNDULANT fever is of common occurrence along the shores of the Mediterranean and in its islands, and in this region has been generally designated Malta fever. Although from its symptomatology and pathological anatomy it was recognised as a distinct affection, and was described under various names, its precise etiology was unknown till the publication of the researches of Bruce in 1887. From the spleen of patients dead of the disease he cul-

1904 its mode of spread was fully studied by a Commission, whose work demonstrated that goat's milk is the chief source of infection. The disease also occurs in India, China, South Africa, and in some parts of North and South America, its distribution being much wider than was originally supposed.

The duration of the disease is usually long—often two or three months, though shorter and much longer periods are met with. Its course is very variable, the fever being of the continued type with irregular remissions. In profuse perspiration, usually orchitis, while mortality is low—about 2 per cent (Bruce). In fatal cases the most striking post-mortem change is in the spleen. This organ is enlarged, often weighing slightly over a pound, and in a third of the cases the spleen is the chief seat of the infection. In the spleen the organism is found in great numbers, and in the blood it is also present.

Human infections by *B. abortus* have a wide geographical distribution corresponding with that of the infection in animals. In certain cases the disease may also be due to *B. melitensis*, and in some cases to *B. abortus*. Human infections by *B. abortus* have a wide geographical distribution corresponding with that of the infection in animals.

BACILLUS MELITENSIS (*BRUCELLA MELITENSIS*)

MICROSCOPIC CHARACTERS This organism is generally observed in the tissues, e.g. the spleen, as a rounded, oval, or cocco-bacillary structure about 0.4μ in diameter. It usually occurs singly or in pairs, but in cultures short chains are also met with (Fig. 88). The coccid form of the organism originally led to its being designated a micrococcus. In culture, however, its bacillary character is more obvious, and rod-shaped forms about 0.6 to 1.5μ in length may be observed. It stains fairly readily with the ordinary basic aniline stains. In Gram's method it reacts negatively. It is non-motile.

CULTURAL CHARACTERS Growth occurs under aerobic conditions on ordinary media, but for isolation and routine cultivation, *Inter-infusion agar* gives the best results. The optimum temperature is 37°C , but growth occurs

to what has been described in the case of enteric fever. The reaction appears comparatively early, often about the fifth day, and may be present for a considerable time after recovery—sometimes for more than a year. The serum of apparently normal persons may agglutinate *B. melitensis* in low dilution, e.g. 1 in 25 and sometimes 1 in 50. In the disease, however, the agglutination reaction usually occurs with much higher dilutions, e.g. 1 in 500, or over.

Strains are serologically homogeneous and all react to a specific anti-serum, but S→R variation may occur in culture, the R variant being in-agglutinable by an antiserum for the S type. Strains of the rough type have sometimes been designated '*B. para-melitensis*'. The serological characters of the *Brucella* group as a whole are considered later.

Prophylactic Vaccination. The 1904 Commission found that vaccination with dead cultures of the organism confers a certain degree of protection among those exposed to the disease. As a rule, two injections were given, 200 to 300 million organisms being the dose of the first injection and about 400 million the dose of the second. This prophylactic measure has not been sufficiently investigated to warrant any definite conclusions as to its efficacy, and studies of practical immunisation against *B. abortus* have shown that dead cultures are not effective antigens. Vaccines have also been used in the treatment of the disease, but the results have not been such as to establish their value.

Bacteriological Diagnosis. During life the readiest means of diagnosis is supplied by the agglutination test (*vide supra*).

Cultures may sometimes be obtained from the blood by the usual methods. Cultures are most easily obtained from the spleen either during life by spleen puncture or *post mortem*. Plates of liver-infusion agar are inoculated, and incubated aerobically at 37° C. Film preparations may also be made from the spleen pulp and stained with carbol-thionin and by Gram's method. In the identification of the organism agglutination by an antiserum to a known strain would supply confirmatory proof. Details of the differentiation of *B. melitensis* from other *Brucella* group organisms are given later.

Great care must be exercised in working with cultures of *B. melitensis*, as bacteriologists have become infected from such sources in an unusually high proportion of instances.

BACILLUS ABORTUS *BRUCELLA ABORTUS* and *BRUCELLA SUI*

Bacillus abortus presents close biological similarity to *B. melitensis*. It is important in human medicine on account of its frequent occurrence in cow's milk, and the possibility of its causing undulant fever in the human subject. The organism was first described by Bang in 1897 as the cause of bovine infectious abortion. The infection has a wide geographical distribution and has been long prevalent in milch cows, among which at the present time in this country it is exceedingly common. The infection is not limited to cattle, but has been found occasionally in horses, dogs, and certain other mammalian animals, including wild rats on farms and also in fowls. A similar infection occurs of

Brucella suis, the specific name *abortus* being applied to the variety of bovine origin. While closely related to *B. melitensis* and *B. abortus* it shows distinct differences. The features of this organism are dealt with later.

all, pleomorphic, non-mottled, Gram-
to 1.5μ in length and 0.3 to 0.8μ
B. melitensis Longer forms up to

3μ may sometimes be observed on a rich medium. From material in which the organism is likely to be present in a state of purity, e.g. the heart blood of the foetus, it may be cultivated, according to Bang's original method, in the form of a shake-culture in a tube of serum agar. In primary growth the colonies develop after inoculation in a zone about 10 to 20 millimetres below the surface of the medium. This behaviour has been taken as evidence that the organism is micro-aerophilic; Wilson and others have shown, however, that growth is also possible in the presence of the oxygen of the air.

In a culture in an atmosphere containing the presence of 5 to 10 per cent. of this gas along with 20 per cent. oxygen appears to afford the optimum atmosphere for cultivation of most strains of *B. abortus*. Growth occurs in sealed tubes, apparently because carbon dioxide is given off by the organisms and by various culture media and its retention in the tube favours growth (Wilson). The explanation of the characteristic zonal growth, which has been described above, seems to be that in a tube of medium in which a shake-culture has been made any carbon dioxide produced near the surface is lost and growth does not occur, while in the depth of the medium anaerobic conditions restrain growth, but where both carbon dioxide and oxygen are at a suitable partial pressure (i.e. in the zone described above), growth results. It is unknown how carbon dioxide influences growth, but the effect is not apparently due to change in the hydrogen-ion concentration of the medium. After continued cultivation the organism can be grown under aerobic conditions. The colonies are small transparent disks resembling those of *B. melitensis*. On potato it produces a brownish or chocolate-coloured growth similar to that of *B. melitensis*. It has no obvious fermentative action on carbohydrates.

That *B. abortus* is responsible for bovine abortion has been proved by experimental infection in pregnant cows, e.g. by injection of cultures into the vagina or by feeding. Intravenous injection in pregnant rabbits and guinea-pigs may lead to abortion in these animals. Parenteral injection of cultures of *B. abortus* produces an active but non-fatal infection in guinea-pigs associated with necrotic lesions in lymph glands, spleen, and liver. The infection in cattle is usually associated with little general effect. The development of the foetus is arrested, and there may be inflammation of the foetal membranes. The organism may persist in the genital organs for some time after the abortion; it localises in the udder and is discharged in the milk for a varying period, so that the animal becomes a carrier of the infection. The agglutination reaction of the serum with known cultures is applied in the recognition of infected animals, and even low titres, e.g. 1 in 20, are regarded as significant. Specific agglutinins are also demonstrable in the whey of the animal's milk and it has been pointed out that a titre of 1 in 80 or over is strong evidence of udder infection.

DIFFERENTIATION OF *B. MELITENSIS*, *B. ABORTUS*, AND *B. BR. SUI* *B. melitensis* and *Brucella suis* (the porcine type of *B. abortus*) do not exhibit the carbon dioxide requirement for growth of *B. abortus* and can grow under ordinary aerobic conditions. *B. melitensis* does not produce hydrogen sulphide in a medium containing organic sulphur, whereas the other types form this gas for the first four days of growth (p. 384). The porcine type is the more active in this respect. The selective action of the dyes thionin and basic fuchsin, incorporated in plates of culture medium, has also been utilised

for differentiating the three types (Huddleson). The following table summarises the differences:

	Carbon dioxide requirement.	Hydrogen sulphide production	Inhibition of growth by	
			Thionin 1 : 30,000	Basic fuchsin 1 : 25,000
<i>B. melitensis</i>	—	—	—	—
<i>B. abortus</i> , bovine type	+	+	Inhibition	—
<i>Br. suis</i> (<i>B. abortus</i> , porcine type) (American strains)	—	++	—	Inhibition

Methyl violet (1 : 50,000) and pyronin (1 : 100,000) yield results similar to those with basic fuchsin. *Br. suis* strains occurring in Denmark differ from the American variety in failing to produce hydrogen sulphide and in being more susceptible to inhibition by dyes.

Serological and antigenic characters of *Brucella* group. As shown by Evans *B. abortus* and *B. melitensis* are so closely related serologically that they cannot be differentiated by direct agglutination. Quantitative agglutinin-absorption tests, however, enabled the two organisms to be separated. The study of a number of strains showed that the majority of those of bovine and porcine origin were serologically of the *B. abortus* type. One strain of *B. melitensis* type was also isolated from an aborted bovine foetus and another from a goat after abortion. '*B. para-abortus*' strains have been described which can be differentiated from *B. abortus* by direct agglutination tests, and are analogous to '*B. para-melitensis*'. These are rough variants of the typical forms. The 'roughness' of strains can be ascertained by the thermo-agglutination test, i.e. boiling in saline for two hours.

The relationship of the different members of the *Brucella* group has been well represented in a study of their antigenic structure by Wilson and Miles. They have postulated two main antigenic constituents (*A* and *M*). In *B. abortus*, *A* predominates quantitatively; in *B. melitensis*, *M*; while the American porcine type is intermediate, *A* predominating but to a lesser degree than in the bovine variety. It seems likely from further studies of strains of these organisms that there are other intermediate types each possibly characteristic of a particular locality, some classified as *B. melitensis*, others as *B. abortus*. Thus a Rhodesian type derived from cattle and classified as *B. abortus* has certain characters in which it resembles *B. melitensis* (e.g. ability to grow without carbon dioxide) and it is apparently of high virulence to the human subject.

A considerable amount of attention has been directed to chemical studies of the constituents of the *Brucella* group organisms in relation to their antigenic and toxic properties, but chemical fractionation of cultures has not so far yielded any particularly noteworthy results.

It is noteworthy from experiments that *B. abortus* killed by heat whereas considerable success in producing immunity to infectious abortion has been attained by inoculation with attenuated living cultures. Recently Huddleson (1942) has obtained from live *Brucella* cultures a water-soluble antigen which is destroyed by antiseptics and by heat. The effective antigen is clearly a very labile substance.

The toxin of the *Brucella* group is of the nature of an endotoxin and it also appears to be markedly thermolabile. Toxicity can be demonstrated by injecting large doses of culture into small animals and these may produce a lethal effect.

Relationship to Undulant Fever in Man. The occurrence of *B. abortus* in undulant fever ('abortus fever') of the human subject is now well recognised and cases have been recorded in various countries including Great Britain. The infection may occur in persons whose occupation brings them into contact with diseased animals, e.g. abattoir workers, farmers, etc., and the organism can readily invade the body through abraded skin, but many cases of 'abortus fever' are undoubtedly due to infection from cow's milk, and it has been clearly shown that *B. abortus* is present in a considerable proportion of samples of raw market milk. It is, in fact, remarkable that the infection is not more prevalent in view of the occurrence of the organism in cow's milk. In this connection it may be noted that young subjects are relatively immune and in any case the average susceptibility of adults to the bovine strains must be comparatively low. The illness varies considerably in its general symptoms, most cases due to the bovine strains are mild, and a proportion may be ambulatory. The fever is undulant in type and is associated with profuse night sweats and often pains in the joints. Arthritis, orchitis, and other localised lesions may occur as complications. In certain cases in which the disease has occurred in pregnant women abortion has resulted (Hasseltine). Morales-Otero records experiments in which human volunteers became infected as the result of drinking milk, containing a strain of the organism, on repeated occasions, or inoculating an area of abraded skin once with the organism, positive results were got with *B. melitensis* and with both bovine and porcine types of *B. abortus*, by both routes in each case. Certain accidental infections seem to have been contracted by inhalation. The diagnosis rests on the results of bacteriological examination. Sometimes the organism may be isolated by blood culture, but this method often gives negative results even in undoubted cases. The agglutination reaction with the patient's serum and known strains of *B. abortus* generally offers a reliable means of diagnosis, and the titre is frequently high, e.g. 1 in 1,000 to 1 in

signs, and it has been suggested from the results of agglutination tests with sera submitted to laboratories for other examinations, that over 1 per cent of the general community in this country may be infected.

Brucella suis is also responsible for undulant fever in the human subject, the infection being usually derived through contact with pigs or their carcases, as in the meat-packing industry in the United States, though it has been shown that the organism may sometimes be transmitted to dairy cows and then conveyed to man by milk. In America this organism produces a more severe illness than *B. abortus* and strains possess a higher virulence than the latter. This, however, is not the case with strains occurring in Denmark.

It would appear that of the various types of *Brucella*, *B. melitensis* possesses the highest pathogenicity, *B. abortus* the least, while the American *Br. suis* is intermediate between the other two. The Danish strains of this type are apparently less virulent even than *B. abortus*. Though some workers have drawn distinctions among these organisms as regards their pathogenic effects in experimental animals it is doubtful if essential differences exist.

Bacteriological Diagnosis. Growth in an atmosphere with 5 to 10 per cent. of carbon dioxide added, provides the most convenient means of obtaining primary cultures, e.g. from the stomach contents of an infected calf; these should be kept at 37° C. for seventy-two hours or longer. The required concentration of carbon dioxide to yield surface growths in subcultures may be obtained by flaming the cotton-wool plug in the culture tube for two or three seconds, then pushing it in and fifteen seconds later closing the tube with an indiarubber stopper or sealing it (Wilson). When primary cultures are required from material likely to contain other organisms, e.g. from the genital passages of infected cows, cultivation presents difficulty and requires special methods. A liver-infusion agar (to which may be added crystal violet 1 : 1—200,000) has been found most suitable. For the demonstration of *B. abortus* in milk, the inoculation of a guinea-pig may be resorted to. A male animal of 300 to 600 grms. weight is used its blood serum being

used in a mixed infection). The animal is killed after six weeks and its serum tested for *Brucella* agglutinins, cultures are made from the urine and also by rubbing the cut surface of various organs (lung, liver, spleen, kidney, testes, and enlarged lymph glands) on the surface of plates of crystal violet liver-infusion agar. In infected animals the spleen is enlarged even up to six times the normal size and is studded with nodules, except in the most acute condition. The epididymus, or less often the body of the testis, shows abscesses. In *Br. suis* (American type) infections the lesions may reach a size of 1 to 2 cm. Direct cultures may also be made from the cream which separates after specimens have been kept for twenty-four hours in the refrigerator (Huddleson *et al.*).

The agglutination test in the diagnosis of the animal infection has been referred to earlier and also methods and criteria for the identification of the different types of *Brucella*.

In the diagnosis of undulant fever due to *B. abortus* the procedures applicable to infection by *B. melitensis* (q.v.) may be adopted. Blood cultures, however, should be incubated in an atmosphere containing 10 per cent. of carbon dioxide. Generally the agglutination test with patient's serum serves to establish the diagnosis, particularly a rising titre on repeated testing. A complement-fixation test has also been used by some workers with satisfactory results, the claim being made that it is more sensitive than the agglutination test. Another sero-diagnostic method is the 'opsonocytophagic' test (Huddleson *et al.*, 1933). This consists in mixing citrated blood with a heavy suspension of *B. abortus* and after thirty minutes at 37° C. making films, which are then suitably stained; the number of phagocytosed organisms in twenty-five polymorphonuclear cells is counted and the phagocytic index determined, i.e. the average number per leucocyte. An index over forty and the presence of organisms in 40 to 60 per cent. of the cells has been regarded as indicative of infection. This reaction, however, often develops later than the agglutination reaction and, moreover, its diagnostic specificity has been questioned. An allergic skin reaction has also been applied in diagnostic work, preparations from cultures of *B. melitensis* or *B. abortus*, 'Brucellin' and 'Brucellergen', are injected intracutaneously. This reaction generally develops later than the agglutination reaction, and it is doubtful if it offers as satisfactory a diagnostic test as the latter.

CHAPTER XVIII

THE INFLUENZA BACILLUS, BACILLUS PERTUSSIS, AND ALLIED ORGANISMS

BACILLUS INFLUENZÆ (*HÆMOPHILUS INFLUENZÆ*)

THE first accounts of the organism known as the influenza bacillus were published simultaneously by Pfeiffer, Kitasato, and Canon, in 1892. The two first-mentioned observers found it in the bronchial sputum, and obtained pure cultures, and Canon observed it in the blood in a few cases of the disease. This organism, up to the time of the influenza pandemic of 1918, was generally accepted as the primary causal agent, although proof was wanting. As a result of the 1918 pandemic, the etiology of the disease was extensively re-studied, but opinions varied as to whether this organism represented the primary agent or only an associated and secondary infection. However, recent work has indicated that the disease is primarily due to a filterable virus. This subject will be discussed in Chapter XXX. The pathogenic properties of the influenza bacillus and its relationship to the epidemic disease, will be dealt with in the present chapter.

MICROSCOPIC CHARACTERS. The bacilli as seen in the sputum are very minute rods not usually exceeding $1.5\ \mu$ in length and $0.3\ \mu$ in thickness. (Fig. 89). They are straight, with rounded ends, and sometimes stain more deeply at the extremities. The bacilli occur singly, in pairs, or form clumps by their aggregation. They take up the basic aniline stains somewhat feebly, and are best stained by a weak solution (1 : 20 to 1 : 200) of carbol-fuchsin applied for five to ten minutes. They are Gram-negative, non-motile, and do not form spores. According to Pittman the influenza bacillus in its primary virulent

phase is capsulate, but as a rule strains which have been recently isolated fail to show a capsule. It must be recognised that the organism tends towards pleomorphism, and some strains show elongated thread-like forms which may even be the predominant morphological type. Certain strains may also exhibit spherical forms measuring 2 or $3\ \mu$ in diameter. Sometimes the filamentous forms show large globular or fusiform expansions. While strains recently isolated from influenza cases usually exhibit the short bacillary form, after artificial culture they tend to become atypical and to show morphological variation, which may be associated with changes in colony form, *i.e.* the transformation from the S to R type (*vide infra*). In the early stages of the more acute cases with catarrhal complications of the respiratory system, influenza bacilli are frequently present in large numbers and may be easily found. On the other hand, it is often difficult or impossible to find them even when the symptoms are severe.



FIG. 89. Film preparation of influenza sputum, showing influenza bacilli along with leucocytes. $\times 1,000$.

CULTURAL CHARACTERS. An outstanding biological feature of the influenza bacillus is its inability to grow on ordinary media and its strict requirement of certain growth factors derived from blood; hence the designation 'hæmophilic' applied to it, and the generic name *Hæmophilus*. One of the best media for the growth of the organism is agar containing heated blood. Pfeiffer originally used blood-smeared agar. He had obtained growths of the bacilli on agar which had been smeared with influenza sputum, but subcultures on ordinary agar media or serum failed to grow. Considering the growth in the first cultures to be probably due to the presence of certain organisms...

constituent of the red cells of the sheep, ox, and goat, renders their blood inhibitory, while human blood is slightly inhibitory (see Krumwiede and Kuttner). The optimum temperature is 37° C.; the temperature range for growth is somewhat restricted, the minimum being from 20° to 25° C. Growth occurs under aerobic conditions and only to a limited degree anaerobically.

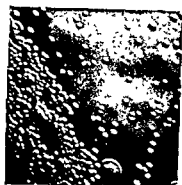


FIG 90. Colonies of influenza bacilli on blood-agar plate. (There are a few larger colonies of other organisms) $\times 5$

The growth of the organism on blood-agar, incubated at 37° C., appears within twenty-four hours, in the form of minute circular colonies, about 0.5 mm. in diameter and almost transparent like droplets of dew (Fig. 90). On the second day they may attain a size of 1 to 1.5 mm., but tend to remain discrete. The colonies do not show any special morphological features; but 'smooth' and 'rough' types have been differentiated. According to Pittman the S type consists of capsulate bacilli (*vide supra*), the R type non-capsulate. The S colonies are relatively large, slightly opaque, somewhat mucoid and iridescent. The R colonies are smaller, more translucent, with a rough granular surface and

indented borders. Growth is favoured by symbiosis; thus, in a mixed plate culture it may be noted that the growth of *B. influenza* is more abundant in the neighbourhood of colonies of other organisms, e.g. staphylococcus, this has been designated 'satellitism'. In blood-broth cultures a thin whitish deposit forms at the bottom of the container.

Owing to its peculiar hæmophilic character *B. influenza* has been carefully studied as regards its requirements for growth in artificial culture. This organism will only flourish in the presence of two growth-promoting principles, which are present in blood (Davis). One of these, designated the X factor, is a highly thermostable substance which remains unaltered even after autoclaving (e.g. at 120° C.). This factor is hæmatin. It was at first supposed that hæmatin acts as a peroxidase in catalysing oxidation by peroxides and so promotes the respiratory functions of the organism.

Certainly it can also serve as an X factor without

On the other hand, these possess

that under anaerobic conditions

the organism can dispense with the X factor (Anderson) and the question therefore arose whether the growth-promoting effect of this factor might depend on catalase action protecting the organism from peroxide. More recent work has clarified the problem and shown that hæmatin probably acts as a growth factor for the influenza bacillus by being utilised for the

phosphopyridine nucleotide (co-enzyme I or II) Thjotta has shown that the V factor is contained in red cells, bacteria, yeasts, and vegetables, and in fact is widely distributed in animal and plant tissues. It is synthesised by various bacteria, *e.g.* staphylococci, and this explains the symbiotic relationship of *B. influenza* to other organisms (*vide supra*).

Certain media have proved specially valuable for the isolation and growth of the bacillus. The so-called 'chocolate' nutrient agar at a temperature of 90° C, *influenzae*, and cultures are somewhat mor on agar containing unaltered blood. It has been supposed that the difference between the altered and unaltered blood depends on the fact that the fresh blood deviates oxygen from the bacillus in virtue of its greater oxygen affinity (Fildes). Another medium particularly valuable for isolating the organism

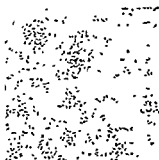


FIG 91. Film preparation from young culture of influenza bacillus. Stained with dilute carbol-fuchsin $\times 1,000$



FIG 92. Film preparation from older culture of influenza bacillus, showing involution forms $\times 1,000$

is agar with a peptic digest of blood added as recommended by Fildes, it has the advantage of being transparent as compared with 'chocolate' agar, which is opaque.

Some strains produce *haemolysis* when growing on blood agar, and it has been noted that these show the atypical coarse bacillary and thread-like forms referred to above, the colonies are also more opaque than the common type, and in fluid medium a flocculent growth results. Many of the haemolytic strains are being independent of the V factor. Strains with similar growth requirements, but lacking haemolytic power (sometimes designated 'para-influenza bacilli'), have been isolated from ulcerative endocarditis.

BIOCHEMICAL REACTIONS Fermentative properties are somewhat irregular. Glucose is generally fermented with acid production, lactose and mannitol are not acted on, galactose and levulose are generally fermented by the non-haemolytic strains, though less frequently by the haemolytic organisms. Saccharose fermentation is a more frequent property of the haemolytic type than the non-haemolytic strains. Fermentation reactions may, however, vary on continued cultivation. Cultures of the influenza bacillus exhibit reducing properties and nitrite is formed from nitrate. A considerable proportion of strains of *B. influenzae* have been found to

THE INFLUENZA BACILLUS

produce indole in culture; others, including the hæmolytic variety, lack this property.

VIABILITY. The powers of resistance of this organism are of a low order. Pfeiffer found that dried cultures kept at the ordinary temperature were usually dead in twenty-four hours, and that if sputum were kept in a dry condition for two days cultures could be no longer obtained. From these experiments it follows that outside the body in ordinary conditions they can remain alive for only a short time. Cultures show feeble viability, dying within a few days; to keep strains alive subcultures must be made every four to five days. Heating at 50° to 55° C. kills the organism in thirty minutes.

SEROLOGICAL CHARACTERS. The strains may even exhibit serological characters. This is analogous to what has been observed in the *B. coli* group. It has been pointed out, however, by Pittman in a study of 'smooth' capsulate strains of *B. influenza* that these can be classified into six serological types, their specificity depending apparently on a capsular carbohydrate substance. Such strains on transformation to the 'rough' form lose their original serological characters and show the extreme antigenic heterogeneity referred to above.

Distribution in the Body. The bacilli are found chiefly in the respiratory passages in influenza. They may be present in large numbers in the nasal secretion, generally mixed with a considerable number of other organisms, but it is in the small masses of greenish-yellow sputum that they are present in greatest numbers. They occur in large numbers within the leucocytes, and towards the end of the disease a large proportion have this position. They may persist for weeks after symptoms of the disease have disappeared, and may still be detected in the sputum. Especially is this the case when there is any chronic pulmonary disease. They occur also in large numbers in the capillary bronchitis and catarrhal pneumonia of influenza, as Pfeiffer showed by means of sections of the affected parts. In these sections he found the bacilli lying amongst the leucocytes which filled the minute bronchi, and also penetrating between the epithelial cells and into the superficial parts of the mucous membrane. McIntosh has shown that at a very early stage the bacilli are present in the upper respiratory tract but absent from the lung. Other organisms also, e.g. streptococci, pneumococcus, may be concerned in the pneumonic conditions following influenza. Occasionally in the foci of suppurative softening in the lung the influenza bacillus has been found in a practically pure condition. In cases of empyema the organisms present would appear to be chiefly streptococci and pneumococcus, but the influenza bacillus may also be present, whilst in the gangrenous conditions which sometimes occur a great variety of organisms has been found.

Pfeiffer's observations on a large series of cases convinced him that the organism was very rarely present in the blood—that in fact its occurrence there must be looked upon as exceptional. The conclusions of other observers have, on the whole, confirmed this statement. The bacillus may be present in other lesions complicating influenza. Pfeiffer found it in inflammation of the middle ear, and it has been observed in meningitis following influenza. Pfuhr considered that in these cases the path of infection is usually a direct one through the roof of the nasal cavity. He also found *post mortem*, in a

rapidly fatal case with profound general symptoms, influenza bacilli in various organs, both within and outside the vessels. In a few cases also the bacilli have been found in the brain and its membranes, with little tissue change in the parts around.

The frequent association of the organism with epidemic influenza has been well established, but it is not invariably present. Its occurrence is, moreover, specially related to the catarrhal complications. During the 1918 pandemic, cases were met with in which the fever and general manifestations were exceedingly marked but unaccompanied by marked inflammatory involvement of the respiratory tract, and *B. influenza* was not detectable in the nose and throat secretions. Even in cases with pulmonary complications, the organism could not be found constantly in the sputum when examined by the best methods. Such observations led to the view that, contrary to previous belief, this organism is not the primary agent in epidemic influenza, though it was admitted that it represents a frequent concomitant infection and may be responsible, like streptococci and the pneumococcus, for pulmonary complications.

Extensive observations on the bacteriology of the respiratory system show that the influenza bacillus may be present in a great variety of conditions apart from epidemic influenza. It has been obtained from the fauces, bronchi, and lungs in various inflammatory conditions, and also in specific fevers. It is not infrequent in phthisis, in bronchiectatic cavities, and in chronic inflammation of the nasal sinuses. It may be present in pure culture in suppuration of a joint, especially in children. This organism may also be recovered frequently from the nose and throat in quite healthy persons and has a place among the commensal flora of the respiratory passages.

A hæmophilic organism has been not infrequently found in cases of acute primary meningitis occurring in young subjects, *e.g.* from two to three years of age. In the cerebro-spinal fluid this organism may appear as somewhat elongated filaments, and it was originally thought to be of the nature of a leptothrix. It is, however, indistinguishable from the influenza bacillus, and the filamentous form is not a constant character. This organism differs from the usual strains of *B. influenza* in its higher virulence for laboratory animals (Rivers).

Experimental Inoculation. Pfeiffer, by injecting living cultures of the organism into the lungs of monkeys, in three cases produced a condition of fever of a remittent type. There was, however, little evidence that the bacilli had undergone multiplication, the symptoms being apparently produced by their toxins. He accordingly came to the conclusion that the influenza bacillus contains toxic substances which can produce in animals some of the symptoms of the disease, but that animals are not liable to infection, the bacilli not having the power of multiplying to any extent in their tissues. Wollstein distinguished virulent and avirulent types according to the result on intravenous injection in the rabbit: the virulent types caused death in about twenty-four hours, used, however, was complete for a rabbit of 1,000 grms. Strains from the respiratory tract were non-virulent by this test, those from the blood and meninges, and rarely from pneumonic lung, were virulent. It has been found that the virulence of the influenza bacillus in animal experiments can be enhanced by injecting cultures along with mucin, as is the case with various other organisms, *e.g.* meningococcus. Wollstein found that a fatal cerebro-spinal meningitis could be produced in monkeys by the subdural injection of virulent cultures. McIntosh claimed that *B. influenza* produces a diffusible toxin, and

that inoculation of animals, *e.g.* rabbits and guinea-pigs, with filtered cultures leads to pathological changes in the lung which show a close resemblance to those observed in man. This toxic substance is not to be regarded as a true exotoxin. Cecil and Steffen, by introducing virulent influenza bacilli into the throat and nose in the human subject, produced an acute catarrhal infection of the upper respiratory passages, which, however, fell short of typical influenza.

Reviewing the extensive experimental work that has now been carried out with *B. influenzae*, we may say that conclusive proof of its primary etiological relationship to the disease has not been obtained. On the other hand, the observed relationships of the organism to lesions in the lungs and elsewhere leave no room for doubt that it is possessed of pathogenic properties.

The question of the virus etiology of influenza and the pathogenic association of a filterable virus and *B. influenzae* is discussed in Chapter XXX

Bacteriological Diagnosis. (a) *Microscopic* A portion of the greenish-yellow purulent material which often occurs in little round masses in the sputum should be selected, and film preparations should be made in the usual way. Films are best stained by dilute carbol-fuchsin (1:20 to 1:200), staining being prolonged for five to ten minutes. In sections of the tissues, such as the lungs, the bacilli are best observed, as shown by Pfeiffer, by staining with the same solution as above for half an hour. The sections are then placed in alcohol containing a few drops of acetic acid, in which they are dehydrated and slightly decolorised at the same time. They should be allowed to remain till they have a moderately light colour, the time varying according to their appearance. They are then washed in pure alcohol, cleared in xylol, and afterwards mounted.

(b) *Cultures* A suitable portion of the greenish-yellow material having been selected from the sputum, it should be washed well in several changes of sterilised water. A loopful should then be used to make successive strokes on the surface of blood agar plates. Heated-blood ('chocolate') agar or Fildes' peptic blood digest agar are specially useful for isolating the organism. The plates should be incubated at 37° C, when the transparent colonies of the influenza bacillus will appear, usually within twenty-four hours. These should fail to grow in subculture on ordinary agar media. Penicillin is most valuable as a selective bacteriostatic agent in isolating *B. influenzae*. It inhibits the growth of the Gram-positive organisms present often in large number in the sputum. It is incorporated in the medium or spread on the surface (0.5 unit per c. c. of medium).

The requirement of the X and V factors can be determined by serial cultural tests in peptone water (1) *plus* hæmatin (X), (2) *plus* yeast extract (V), and (3) *plus* both hæmatin and yeast extract.

ORGANISMS CLOSELY RELATED TO THE INFLUENZA BACILLUS

Para-influenza Bacillus (*Hæmophilus para-influenzae*) Reference has already been made to this organism. Strains differ from the typical influenza bacillus in being independent of the X factor, though requiring the V factor; they consistently ferment saccharose and maltose; only a few strains produce indole. Hæmolytic strains have been included under this designation. The para-influenza bacillus has been described mainly in acute pharyngitis. strains have also been found in ulcerative endocarditis.

Bacillus influenzae suis (*Hæmophilus influenzae-suis*). This organism has been isolated from swine influenza, but in this disease it is associated with a

filterable virus related to the virus of human influenza. It differs from the typical influenza bacillus only in its lack of fermentative properties and its inability to produce indole (Lewis and Shope), but it is doubtful whether these negative features clearly differentiate it from *B. influenzae*.

Bacillus hæmoglobinophilus canis (*Hæmophilus canis*) was originally

colonies on a blood medium tend to be larger and more opaque than those of the influenza bacillus and it also differs clearly from this organism in fermenting mannitol. It ferments glucose and saccharose and produces indole. There is no evidence of its possessing pathogenic properties. (An organism with the same requirements as regards the X and V factors, but with the



FIG 93 Film preparation from a case of acute conjunctivitis, showing Koch-Weeks bacilli, chiefly contained within a leucocyte (From a preparation by Dr Inglis Pollock) $\times 1,000$



FIG 94 Koch-Weeks bacillus, from a young culture on blood agar. Stained with dilute carbol-fuchsin. $\times 1,000$

additional requirement of carbon dioxide for its growth has been described in endocarditis—Khairat.)

Koch-Weeks Bacillus (*Hæmophilus conjunctivitis*) This organism has long been recognised as an important causal agent of acute contagious conjunctivitis. It can readily be found in the muco-purulent secretion by staining films with dilute carbol-fuchsin and is often seen in the interior of leucocytes (Figs 93, 94). In all its biological characters it is indistinguishable from the influenza bacillus, and its separate designation hardly seems justifiable.

As regards their requirement of the X and V factors the influenza bacillus and closely related organisms may be classified as follows

	X	V
<i>B. influenzae</i> (including Koch-Weeks bacillus and <i>B. influenzae suis</i>)	+	+
<i>B. para-influenzae</i>	—	+
<i>B. hæmoglobinophilus canis</i>	+	—

DUCREY'S BACILLUS OF SOFT SORE (*HÆMOPHILUS DUCREYI*)

The bacillus of venereal soft sore (chancroid) was first described by Ducrey in 1889, who found it in the purulent discharge from the ulcerated surface, and later, in 1892, Unna described its appearance and distribution as seen in sections through the sores.

MICROSCOPIC CHARACTERS. The organism occurs in the form of minute oval rods measuring about 1.5μ in length, and 0.5μ in thickness and sometimes showing bipolar staining (Fig. 95). It is found mixed with other organisms in the purulent discharge from the surface of the lesions, and is chiefly arranged in small groups or in short chains. When studied in sections through the ulcer, it is found in the superficial part of the floor, but more deeply situated than other organisms, and may be present in a state of purity in the leucocytic infiltration. In this position it is usually arranged in chains, which may be of considerable length, and which are often seen lying in parallel rows between the cells. The bacilli chiefly occur in the free condition.

In many cases the organism is present in the associated buboes in a state of purity; it has been found there by microscopic examination, and cultures



FIG. 95. Ducrey's bacilli.

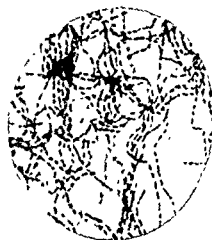


FIG. 96. Ducrey's bacillus from a twenty-four-hour's culture in blood broth $\times 1500$.

have also been obtained from this source. Sometimes the ordinary pyogenic organisms become superadded.

The bacillus in film preparations can readily be stained by most of the ordinary dye solutions, though Löffler's methylene blue solution is preferable, as it does not overstain. In sections, however, great care must be taken in the process of dehydration, and the aniline-xylol method should be used for this purpose, as alcohol decolorises the organism very readily. Ducrey's bacillus reacts negatively in Gram's method. It is non-motile and non-sporing.

In culture it shows appearances similar to those observed in the tissues, but occasionally long undivided filaments are observed. Within a comparatively short period cultures undergo marked involution and great irregularities of form and shape are to be found.

CULTURAL CHARACTERS. The organism is an aerobe and the optimum temperature is about 35°C . Some writers have suggested that the organism grows best under a reduced oxygen tension; it has also been pointed out that growth is favoured by increased carbon dioxide in the atmosphere, as in sealed culture tubes. Ducrey's bacillus may be definitely classified with the *Hæmophilus* group, as it has been shown to require the X factor though independent of the V factor. In this respect it resembles *Hæmophilus canis*. A comparatively large amount of blood is needed for primary culture.

¹ We are indebted to Dr. Davis for the use of Figs. 95 and 96.

Bezançon, Griffon and Le Sourd obtained pure cultures on a medium consisting of rabbit's blood and agar, in the proportion of one part of the former to two of the latter. The blood was added to the agar in the melted condition at 45° C., and the tubes were then sloped. Davis confirmed these results, and found that another good medium is freshly drawn human blood distributed in small tubes, this method is specially suitable, as the blood inhibits the growth of various extraneous organisms. Initial cultures,

cultured on blood agar. Cultures may be made by this method from the buboes, the inoculum being obtained by puncturing the enlarged gland with a syringe. On solid medium the growth appears in the form of very small round confluent colonies.

A¹ even traces of hæmatin in nutrient broth serve as X factors for the growth of the organism.

Relations to the Disease, Experimental Inoculation, and Immunity Reactions. Subcutaneous or intravenous inoculation of the ordinary laboratory animals is not attended by any result, but it has been found that some monkeys are susceptible, small ulcerations being produced by superficial inoculation, and in all these the organism can be demonstrated. Rabbits can also be inoculated intradermally, an inflammatory indurated lesion results followed by necrosis, the lesion heals spontaneously (Feiner and Mortara). Tomaszewski cultivated the organism for several generations, and reproduced the disease by inoculation of the human subject. The causal relationship of this bacillus must therefore be considered as completely established, and the conditions under which it grows show it to be a strict parasite under natural conditions—a fact which is in conformity with the known data as to the transmission of the disease. A method of demonstrating the presence of the organisms where they are scanty consists in injecting pus or secretion from the patient intracutaneously into himself, after two or three days Ducrey's bacillus can often be found in the contents of the resulting lesion (Ravaut, Rabreau and Hesse). A cutaneous allergic reaction can be elicited in infected persons by intradermal injection of the killed organisms. This reaction shows a high degree of specificity (Reenstierna). Strains of the organism are agglutinated by specific antisera and this reaction may be applied for purposes of identification.

BACILLUS PERTUSSIS (*HÆMOPHILUS PERTUSSIS*)

Whooping-cough is now in many countries one of the most important epidemic diseases of childhood, in addition to causing a considerable death-rate, it also leads to serious impairment of resistance to other infections.

Up to the year 1906, the chief result of bacteriological observations, of which those of Spengler, Jochmann and Krause, and Davis may be mentioned, had been to demonstrate the very frequent presence of minute hæmophilic bacilli resembling the influenza bacillus in the sputum and also in the lesions in this disease. In that year, however, Bordet and Gengou published an account of another minute organism, designated *Bacillus*

pertussis, and brought forward certain facts which gave strong support to its etiological relationship.

MICROSCOPIC CHARACTERS. The organism, as seen in the sputum, occurs in the form of minute bacilli with a distinct tendency to the cocco-bacillary form. It is about the same size as the influenza bacillus. It stains rather faintly with ordinary stains, and the margin and extremities are often more deeply coloured than the centre, which may appear as an uncoloured spot, it is Gram-negative, non-motile, and does not form spores. In cultures the bacilli present the same characters, and are distinctly less pleomorphic than the influenza bacillus (Fig. 97). They are specially numerous at the beginning of the disease, and they may be found in large numbers in the opaque whitish sputum expectorated from the bronchi. As the disease advances they become scanty, and may disappear when the symptoms of the disease are still prominent.



FIG. 97. Film preparation from a twenty-four hours' culture of the bacillus of whooping-cough. Stained with dilute carbol-fuchsin $\times 1,000$.

CULTURAL CHARACTERS. Bordet and Gengou succeeded in obtaining pure cultures on the glycerol-potato-blood-agar medium described in the Appendix, and this was found to be the most suitable of all the media tried. For primary cultivation a high proportion (e.g. 50 per cent.) of fresh blood in the medium is essential. The optimum temperature is about 37° C. and the organism is a strict aerobe. In the first cultures, growth consists of characteristic minute, raised, somewhat opaque, 'pearly' colonies; later it becomes much more abundant, and subcultures may be made on ordinary blood or serum agar media. Confluent

growths have been likened to smears of aluminium paint. As compared with that of the influenza bacillus, growth after several generations is thicker and more opaque and the margins are more sharply marked off; it also has a tenacious character. Primary growths occur more slowly than those of the influenza bacillus. The presence of blood, though favouring the growth, is not so essential as in the case of the influenza bacillus, and *B. pertussis* can grow independently of the X and V factors (p. 388). It does not ferment glucose or other carbohydrates and does not produce indole or reduce nitrate (cf. *B. influenzae*). It is non-hæmolytic. It may be noted that *B. pertussis*, unlike the influenza bacillus, will not grow in primary culture on a heated-blood medium.

VIABILITY. The resistance of *B. pertussis* is somewhat similar to that of the influenza bacillus, but cultures have much greater viability at low temperatures, e.g. 0° to 10° C.

SEROLOGICAL CHARACTERS. Bordet and Gengou found that in every case examined the serum of convalescents gave the complement-fixation reaction very markedly with the whooping-cough bacillus, which shows that a specific antibody for the bacillus is present in the serum, and points to a true infection with the organism. The results of the application of the test to adults suffering from bronchial irritation have been to show that they more frequently suffer from the infection than was formerly supposed, the paroxysmal stage being often absent.

It is specially noteworthy that this organism constitutes a homogeneous species from the serological point of view and contrasts in this respect with the influenza bacillus. Thus most observers have found on testing the agglutination reactions of freshly isolated strains with antisera that they all react similarly. On the other hand, after cultivation, strains rapidly undergo antigenic variation. This seems to be analogous to the S→R transformation, though there is no distinct change in colony structure. Certain serological observations indicate that the change in antigenic characters develops in successive phases (Leslie and Gardner, Toomey *et al.*). Leslie and Gardner have recognised four antigenic types representing these phases, which they designated I to IV. It has also been pointed out that the primary S form is capsulate and there is some evidence that the capsular substance is of carbohydrate composition (Lawson, Cruickshank and Freeman, Flosdorf and Kimball).

Pathogenic Effects, Relationship to Disease and Toxin. The general results obtained by Bordet and Gengou indicated that the ordinarily used animals were not susceptible to true infection with the bacillus, but that it contained a powerfully acting toxin, which produced both local and general effects. The injection of a small quantity of the bacillus into the eye of a rabbit produced a local necrosis, with little inflammatory change, and the introduction of dead, as well as living, cultures into guinea-pigs caused death from toxic action, there being hæmorrhagic œdema locally, and hæmorrhages and necrotic foci in organs. They advanced the view that the bacillus is present in

whooping-cough concluded that the essential feature consists in the presence of masses of the bacilli between the cilia of the epithelial cells lining the

irritation and irregular pyrexia were outstanding features. Usually, in the case of the dogs, a fatal result followed after two or three weeks, and *post mortem* there were found catarrhal changes in the respiratory tract and sometimes patches of broncho-pneumonia, from which the bacillus could be recovered in pure culture. The serum of the infected animals gave the complement-fixation reaction. A specially interesting finding was that a number of healthy young dogs contracted the disease by contact with the inoculated. Fraenkel also obtained results closely similar to those of Klimenko. In recent years further attempts have been made to establish the etiological rôle of *B. pertussis* by experimental inoculation. Sauer and Hambrecht introduced cultures into the nares and larynx of young monkeys (e.g. *Macacus rhesus*) and in certain cases after an incubation period of one to three weeks paroxysmal coughing resulted. Examination of the blood revealed the transitory lymphocytosis which is characteristic of the human disease. *B. pertussis* was recovered by throat culture during life and from the lungs *post mortem*. Recovered animals were immune to subsequent inoculations. Intranasal inoculation of anaesthetised mice has been found to produce an interstitial pneumonia with a histological picture somewhat similar to that in the human disease (Burnet and Timmins). Young rats, anaesthetised with ether, are also susceptible to intranasal inoculation, with a resulting interstitial pneumonia; these animals develop a paroxysmal cough which may persist for long periods in those which survive the infection (Hornbrook and Ashburn). Shibley and Hoelscher claim to have reproduced

the disease in chimpanzees. Experiments have also been carried out in chimpanzees by Rich and his co-workers; these demonstrated the infectivity of cultures of *B. pertussis* and of unfiltered material from the respiratory passages of cases of whooping-cough. The conditions produced closely resembled whooping-cough. No evidence was obtained that any filterable infective agent is responsible for the complete syndrome of effects which is typical of whooping-cough, though there were some indications of the presence in experimentally inoculated animals of a transmissible filterable catarrh-producing agent. The production of whooping-cough in children by means of cultures of *B. pertussis* has been recorded (Macdonald and Macdonald).

As mentioned above, marked toxic effects were observed by Bordet and Gengou in animals inoculated with living or killed cultures of *B. pertussis*. Toxic products can be obtained by grinding dried killed cultures and extracting them with saline (Evans and Maitland); the toxin has also been got in filtrates of six-days-old fluid cultures (Roberts and Ospeck). On intravenous injection into rabbits the toxin is lethal, the animals dying with

glycaemia occurring before death, on results. Guinea-pigs and mice are also affected. It is of further interest that when administered intratracheally it produces histological changes in the lungs which are similar to those seen in the human disease—perivascular and peribronchiolar accumulation of lymphocytes, presence of mononuclear cells in the alveoli and areas of necrosis (see Evans, Sprunt and Martin, Ospeck and Roberts; Ehrich *et al.*). The fresh toxic extract and the product detoxicated with formol are antigenic and develop an antitoxin in animals. Heating at 55° C. for half an hour destroys these antigens and abolishes the lethal and necrotising action of the toxin and the property of producing toxic extract with saline.

It is not clear how far the toxin accounts for the features of the human disease.

The question has been considered whether a virus may be associated with *B. pertussis* in whooping-cough, and in this connection intranuclear inclusion bodies have been described in the alveolar epithelium in the disease. The infiltration of the lung tissues with mononuclears and lymphocytes is a feature which provides an analogy with virus infections of the respiratory tract such as measles and influenza, though it has been shown that these changes may be produced by the bacterial toxins. The extremely infectious nature of the disease and the lasting immunity following recovery have also suggested the possibility of whooping-cough being of virus origin. So far, however, attempts to demonstrate a specific virus have been negative or inconclusive.

Sugare and McLeod, who isolated *B. pertussis* from a series of cases of whooping-cough, obtained successful results in every instance on examination in the first week of the disease. They failed to find the organism in secretions from the larynx, trachea, bronchi, and lungs of fifty children under ten years of age who had died from diseases other than whooping-cough. It should be noted that while the organism may be detected in most cases during the early catarrhal stage, it is less regularly found in the expectoration during the paroxysmal stage and may be absent at a later period. Thus, as a rule, cases are non-infective after four weeks from the onset of the typical paroxysmal coughing (Kristensen). The bacillus has not been found in the blood unless as an agonal phenomenon (Klimenko).

All the evidence available so far definitely points to *B. pertussis* as the specific and primary cause of whooping-cough

Immunity. In recent years extensive studies have been made of active immunisation against the disease by means of vaccines. In general such vaccines have consisted of suspensions of recently isolated virulent cultures in the antigenic phase I (Leslie and Gardner), killed and preserved with 0.5 per cent. phenol, and standardised to contain 10,000 to 20,000 million organisms per c.c.; two to four doses have been injected at intervals representing usually a total of 70,000 to 80,000 million organisms, but some workers have preferred smaller doses, e.g. 12,000 million, while others have used an even larger dosage, e.g. 120,000 million organisms. It has been suggested that less than 50,000 million is ineffective. Alum-precipitated suspensions have also been employed with the object of delaying absorption and producing a sustained stimulus, e.g. two doses each of 10,000 million organisms at a month's interval. In trials of the vaccine many writers have recorded significant differences between vaccinated and unvaccinated groups of children, which otherwise seemed comparable, and their findings indicate that an effective degree of protection is conferred and that when the disease is not prevented it is of a milder type than in unvaccinated children. Some observers, however, have recorded disappointing results or differences between vaccinated and unvaccinated which are not statistically significant. It should be remembered that there are great difficulties in the accurate assessment of the value of immunisation against whooping-cough in virtue of the wide variation in exposure to infection among children in the ordinary community (see Siegel, Sauer; Miller and Faber, Kendrick and Eldering, Doull *et al.*, McLean, Bell).

Hæmophilus paraptussis. An organism so designated has been described in cases of clinical whooping-cough (Eldering and Kendrick, 1937). It is differentiated from the classical organism by its production of hæmolysis on the Bordet-Gengou medium and the formation of a brown pigment. Its antigenic composition is apparently different from that of *B. pertussis*, though it shares with the latter organism a minor component. Its toxin is similar to that of *B. pertussis* (see also Brueckner and Evans). A hæmolytic type of *B. pertussis* has also been described by Bradford and Slavin. This organism in subculture grew more profusely than the classical organism and was more rapidly adapted to plain agar. It produced a brown-coloured growth on certain media. Serological differences of a quantitative nature between it and *B. pertussis* were demonstrated.

Bacteriological Diagnosis. The original method was to obtain a portion

plates were then incubated at 37° C. and examined after twenty-four, forty-eight, and seventy-two hours. Colonies of *B. pertussis* appear after seventy-two hours as small raised, pearl-like colonies which can be recognised by their characteristic features.

A procedure which has been extensively applied for obtaining direct cultures of the organism is to hold an open plate of the special medium about four inches in front of the mouth of the patient during the paroxysm of coughing. Fifteen seconds' exposure is sufficient. The plate is then closed and incubated. This method is subject to the drawback that other organisms may overgrow the colonies of *B. pertussis*. However, penicillin added to the medium (0.3 unit per c.c.) is of special value as a selective agent in preventing the growth of other organisms from the respiratory passages, e.g. staphylococci and streptococci.

The use of the post-nasal swab has also been advocated as a more convenient and satisfactory means of obtaining diagnostic material from cases of whooping-cough. The swab is used for inoculating a plate of Bordet-Gengou medium and penicillin is added as a selective agent (Cruckshank).

In infants nasal swabs also give satisfactory results.

B. influenzae and organisms of this type which may occur in sputum require careful differentiation from *B. pertussis*. The strictly hæmophilic character of *B. influenzae* and the features of the growth of *B. pertussis* after several subcultures, as compared with that of the former (*vide supra*), serve to distinguish the two organisms. While *B. pertussis* may be grown after subcultivation on a serum medium, *B. influenzae* completely fails to develop on such media. According to Sugare and McLeod, the chief features which distinguish *B. pertussis* from *B. influenzae* are, in the case of the former, the slowness of growth; the appearance of the colonies on Bordet-Gengou medium, which are raised and pearly; the persistence of viability in cultures after keeping four to five weeks at 10° to 0° C.; and the absence of growth of recently isolated strains on heated blood agar; also the very active catalase effect of cultures, whereas *B. influenzae* gives this only slightly.

The identity of *B. pertussis* can be established rapidly by slide-agglutination with growth from the primary plate culture and specific antiserum.

Serological methods may be used for diagnostic purposes particularly in the later stages of the disease. The agglutination or complement-fixation reaction is suited for the purpose. The precipitin reaction has also been recommended (Farago).

BACILLUS BRONCHISEPTICUS (HÆMOPHILUS BRONCHISEPTICUS)

This organism, which has recently been classified with the *Hæmophilus* group, was included by earlier writers in the *Brucella* group. It was described by Ferry and by McGowan in canine distemper, but constitutes only a secondary invader in this disease. A similar organism occurs in broncho-pneumonia of guinea-pigs and other rodents. *B. bronchisepticus* is similar in morphology to *B. pertussis*, but is motile and possesses peritrichous flagella. It is able to grow on plain agar media though growth is enhanced by the presence of blood. The colonies are small, circular, smooth, glistening, and convex. Hæmolysin is produced in culture. The organism is devoid of saccharolytic properties. In the S phase it possesses an antigenic component in common with *B. pertussis* and its toxin is also similar to that of the latter organism. Moreover, when cultures are inoculated intranasally in mice, lesions result which are similar to those produced by *B. pertussis*. Lesions of the lung in natural infections, e.g. of the rabbit, resemble those of whooping-cough in the human subject.

DIPLOBACILLUS OF MORAX (MORAX-AXENFELD BACILLUS, HÆMOPHILUS LACUNATUS)

This organism, which is specifically associated with conjunctivitis has been included by various systematists in the *Hæmophilus* group, but its characters are such as to render this classification open to serious objection. It has been suggested that a new genus should be recognised for this organism and related species or types, *Moraxella*, and this seems justified.

The bacillus of Morax is especially common in the more subacute cases of conjunctivitis. Eyre found it in 2.5 per cent. of all cases of conjunctivitis. It

is a small plump bacillus, measuring 2 to 3 μ by 1 μ and usually occurring in pairs, or in short chains of pairs (Fig. 98). It is non-motile, does not form spores, and is Gram-negative. In cultures it is distinctly pleomorphic, varying from short cocco-bacillary forms to elongated filaments. It does not grow on the ordinary media, the addition of blood or serum being necessary. The organism is not truly hæmophilic and is independent of the X and V factors of blood (p. 388). Its optimum temperature is 37° C., and it does not flourish at room temperature. On *serum agar* the colonies after twenty-four hours are about 1 mm in diameter, circular, slightly raised, greyish-white, and translucent; on continued incubation they may enlarge and reach a size of 2 to 4 mm in diameter with an opaque centre and more transparent border which tends to be irregular. On *coagulated serum* it forms small rounded colonies which produce pits of liquefaction; hence it has been called the '*Hæmophilus lacunatus*'. Hæmolytic and non-hæmolytic strains have been distinguished by Oag who has also shown that these two types differ in their agglutination reactions with antisera. It is non-pathogenic to the lower animals, but conjunctivitis has been experimentally produced in the human subject by means of cultures.

The *Diplobacillus liquefaciens* or *Bacillus duplex* of Petit is practically identical in microscopic appearances with the bacillus of Morax, and is found in cases of conjunctivitis in which there is often a primary involvement of the cornea. It differs from the bacillus of Morax in being able to grow on ordinary nutrient media and at 20° C. It liquefies gelatin at room temperature. It is antigenically distinct from the bacillus of Morax. A non-liquefying

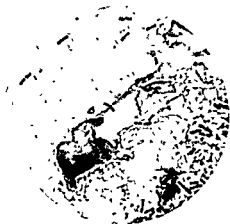


FIG. 98 Film preparation of conjunctival secretion, showing the Morax diplobacillus of conjunctivitis $\times 1,000$

it liquefies gelatin (Jones and Little).

liquefaction (Fig. 102). (It should be noted that considerable variations in the degree and rate of liquefaction of gelatin are observed.) At a later stage liquefaction spreads and may reach the wall of the tube. When the organism is subcultured over a long period of time, it may lose to a large extent the property of liquefying gelatin.

On *gelatin plates* the colonies are somewhat characteristic. They appear as minute whitish points, visible in twenty-four to forty-eight hours, the surface of which under a low magnification is granular. As liquefaction progresses the colonies are able to sink into the medium, leaving a circular depression. In the later stages rings of granular material may be seen around the colonies.



FIG. 102. Stab culture of the cholera vibrio in gelatin—six days' growth. Natural size

Growing on *coagulated blood serum* the cholera vibrio slowly liquefies the medium. On *potato* with a sufficiently alkaline reaction and at a temperature of from 30° to 37° C. a moist layer appears, which assumes a colour varying from yellowish-brown to pink.

In alkaline *broth* the organism grows very readily,

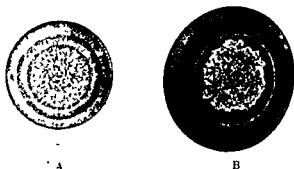


FIG. 103. Colonies of the cholera vibrio on a gelatin plate—three days' growth. A shows the granular surface, liquefaction just commencing, in B liquefaction is well marked.

a general turbidity resulting in twelve hours at 37° C., while the surface shows a granular texture composed of vibrios in a very dense layer.

Growth takes place in peptone solution (1 per cent. with 0.5 per cent. sodium chloride added). Growth occurs on *MacConkey's medium*, the colonies being at first colourless, but later developing a pinkish colour.

On all the media the growth of the cholera vibrio is relatively rapid, and especially is this the case in peptone solution and in broth, a circumstance of importance in relation to its separation in cases of cholera (*vide infra*).

Variation occurs as regards the morphology of colonies, as in other bacterial groups, and 'rough' variants may be observed which are analogous to the 'rough' colonies of the coli-typhoid group. Colony variants have been studied by Balteanu, who has described three types: (1) 'circumvallate rugose' colonies, which are small, yellowish, and opaque; (2) 'ring' colonies, which often show an opaque centre and transparent border; (3) 'opaque' colonies, consisting of non-motile units and in serological tests containing only the O (somatic) type of antigen. According to White the rugose variant produces a capsular substance.

BIOCHEMICAL CHARACTERS. *Vibrio cholerae* produces acid, without gas formation, from glucose, lævulose, galactose, saccharose, mannose, mannitol, maltose, starch, and dextrin; late fermentation of lactose, with acid production, may occur after several days, but different strains show variation in the power of fermenting this sugar. Dulcitol and arabinose are not fermented.

but other vibrios may give the same reactions. However, an unknown vibrio, which yields different results with saccharose, mannose, and arabinose, may be excluded as a true cholera vibrio. In milk the organism grows well, and produces no coagulation nor any change in its appearance, at least for several days. Later, however, acid formation and clotting may occur. *V. cholerae* does not give the Voges-Proskauer reaction while certain other vibrios yield a positive result.

Cholera-red reaction. This is a fairly constant reaction, though it is not peculiar to the cholera vibrio. The test is made by adding a few drops of pure sulphuric acid to a culture in peptone solution (1 per cent) which has been incubated for twenty-four hours at 37° C, a reddish-pink colour of nitroso-indole is produced. This is due to the fact that both indole and nitrite are formed in suitable proportions. Rough variants and strains from carriers may fail to give the nitroso-indole reaction (Seal).

Hæmolytic test. *V. cholerae* has usually been described as non-hæmolytic, whereas other when growing the colonies

cholerae, which are definitely hæmolytic, e.g. the so-called El Tor vibrio (*vide infra*). Further, strains of *V. cholerae* which appear to be non-hæmolytic after twenty-four hours' growth on blood agar, may later produce a clear zone round each colony along with a green discoloration of the blood. It has, however, been found that the hæmolytic test can only be satisfactorily carried out by adding a fluid culture to a blood suspension. Greig recommended the addition of varying amounts, up to 1 c.c. of a three days' culture in alkaline broth, to 1 c.c. of a 5 per cent suspension of goat's corpuscles (sheep's corpuscles may also be used), the whole being made up to 2 c.c., and thoroughly mixed. The tubes are placed in the incubator for two hours at 37° C, and then in the ice-chest overnight, the results being read next day. He found after testing more than 300 strains of true cholera vibrios that none of them produced hæmolysis, whereas this resulted with organisms of the El Tor type referred to. It should be noted that occasional strains of the El Tor vibrio may lose their hæmolytic property after three days' growth. Therefore the cultures tested should not be older than two days. (*V. cholerae* may yield a positive hæmolytic result with blood from other animal species especially after several days' cultivation, e.g. horse, rabbit.)

Reference has been made to the fact that strains of *V. cholerae* may pro-

when these organisms are growing on agar containing heated blood, whereas the El Tor vibrio does not clear this medium. The clearing of heated blood is possibly due to breaking down of hæmoglobin with the formation of alkaline hæmatin. Some vibrios produce both true hæmolysis and this clearing effect (on heated blood), though as shown above the two properties are often dissociated (Finkelstein).

Further reference is made later to the question of hæmolysin production as a feature which distinguishes certain vibrios from the true *V. cholerae*.

Distribution in the Body and Pathological Effects. The vibrios are generally confined to the intestine. But they may be present sometimes in the internal organs, and especially in the gall-bladder and biliary passages. Thus in a large series of post-mortem examinations Greig found the organisms in the gall-bladder in more than a quarter of the cases, and in a considerable number of these distinct pathological changes were present. (Another interesting fact observed by him was that in rabbits inoculated intravenously with the living organisms, infection of the gall-bladder and the formation of gall-stones not infrequently occurred.) An important factor in the pathology of the disease is the toxic effect on the bowel. In cases in which there is the characteristic 'rice-water' fluid, the lower half of the small intestine is the part most affected. Its surface epithelium becomes shed in great part, and the flakes floating in the fluid consist chiefly of masses of epithelial cells and mucus, among which are numerous vibrios. The vibrios also penetrate the follicles of Lieberkühn, and may be seen lying between the basement membrane and the epithelial lining, which becomes loosened by their action. In some very acute cases there may be relatively little desquamation of epithelium, the intestinal contents being a comparatively clear fluid containing the vibrios in large numbers. In other cases of a subacute type, the intestine may show more extensive necrosis of the mucosa and a considerable amount of hæmorrhage into its substance, along with formation of false membrane at places. The intestinal contents in such cases are blood-stained and foul-smelling, there being a great proportion of other organisms present besides the cholera vibrios.

Viability and Modes of Infection. In its resistance to heat, the cholera vibrio corresponds with most spore-free organisms, and is killed in fifteen minutes by a temperature of 55° C., and more rapidly at higher temperatures. Although the vibrio can survive in ice for some time it dies when kept for a few days. Against the ordinary antiseptics it has comparatively low powers of resistance.

As regards its resistance in ordinary conditions, the following facts may be stated. In cholera stools kept at room temperature, the cholera organisms are rapidly outgrown by putrefactive bacteria. Greig found that the period of survival is from one to eight days according to the season and the prevailing temperature, persistence being longer in the colder season and shorter when the temperature was high. The conclusion may also be drawn from the work of various observers, that the vibrios do not multiply in sewage, although they may remain alive for some time. Though the conditions generally favourable for the growth of the cholera vibrio are a warm temperature, moisture, a good supply of oxygen, and a considerable proportion of organic material it is doubtful if it can flourish as a saprophyte. The fact that the

During epidemics the cholera organism has been found in the stools of a considerable number of people suffering from slight intestinal disturbance, and even from the stools of quite healthy individuals; these may be regarded as cholera carriers. Numerous observations, carried out both on convalescents and on contacts having the vibrio in the stools, show that in the great majority of cases it dies out after two or three weeks and usually earlier; it has, however, been detected as long as twelve months after the attack in the stools of some individuals. The organism is usually supplied from the infected individual, and may be transmitted from the infected individual to the healthy individual; these may be regarded as cholera carriers. Numerous observations, carried out both on convalescents and on contacts having the vibrio in the stools, show that in the great majority of cases it dies out after two or three weeks and usually earlier; it has, however, been detected as long as twelve months after the attack in the stools of some individuals. The organism is usually supplied from the infected individual, and may be transmitted from the infected individual to the healthy individual; these may be regarded as cholera carriers.

are necessary before they can be pronounced free. As in the enteric carrier, the organisms apparently persist in the gall-bladder. There is no doubt that carriers play an important part in the spread of the disease, and can originate epidemics.

Cholera organisms are, as a rule, rapidly killed by natural drying, and thus they cannot be spread by air and dust. Cholera is commonly transmitted by means of water or food contaminated by the organism, and there is no doubt that contamination of water supplies by choleraic discharges is the chief means by which communities are rapidly infected. It has been shown that if flies are fed on material containing cholera vibrios, the organisms may be found alive within their bodies twenty-four hours afterwards. Further, Haffkine found that, in a locality infected by cholera, sterilised milk might become contaminated with cholera organisms if kept in open jars to which flies had free access. Thus infection may be carried also by this agency.

Experimental Inoculation. In considering the effects of inoculation with the cholera vibrio, we are met with the difficulty that none of the lower animals suffers from the disease under natural conditions. Accordingly, attempts to induce the multiplication of the organism within the intestine of animals, by artificially arranging favouring conditions, occupied a prominent place in the early experimental work.

Nikati and Rietsch, by injecting the organisms directly into the duodenum of dogs and rabbits, succeeded in producing, in a considerable proportion of the animals, a choleraic condition. These experiments were confirmed by other observers, including Koch. Thinking that probably the vibrio when introduced by the mouth is destroyed by the action of the hydrochloric acid of the gastric secretion, Koch neutralised this acidity by administering carbonate of soda to guinea-pigs, and some time afterwards introduced a pure culture into the stomach. As this method failed to give results, he tried the effect of artificially interfering with the intestinal peristalsis by injecting tincture of opium into the peritoneum, in addition to administering carbonate of soda as before. The result was remarkable, as thirty out of thirty-five treated animals died rapidly with symptoms of general prostration and collapse. *Post mortem* the small intestine was distended, its mucous membrane congested, and it contained a colourless fluid with small flocculi and the cholera organisms in practically pure culture. Koch, however, found that when the vibrios of Finkler and Prior, of Deneke, and of Miller (*vide infra*), were employed by the same method, a certain, though much smaller, proportion of the animals died from an intestinal infection. Though the changes in these cases were not so characteristic, they were sufficient to prevent the results obtained with the cholera organism from being used as a demonstration of the specific relation of the latter to the disease.

Zabolotny found that in the marmot an intestinal infection readily takes place by simple feeding with the organism, there resulting the usual intestinal changes, sometimes with hæmorrhagic peritonitis—the organisms, however, being present also in the blood. Of special interest is the fact, discovered by Metchnikoff, that in the case of young rabbits shortly after birth, a large proportion die of choleraic infection when the organisms are simply introduced along with the milk, as may be done by infecting the teats of the mother. Further, from the animals thus infected the disease may be transmitted naturally to others by contact. In this infection of young rabbits many of the symptoms of cholera were present. But many of these experiments were performed with the Massauah vibrio which is now admitted not to be identical with the classical cholera organism, others with a cholera vibrio obtained from the water of the Seine.

Experiments performed by direct inoculation also supply interesting facts. *Intraperitoneal* injection in guinea-pigs of even minute doses of virulent culture is followed by general symptoms of illness, the most prominent being distension of the abdomen, subnormal temperature, and, ultimately, profound collapse, and death in about twenty-four hours. There is peritoneal effusion, which may be comparatively clear, or may be somewhat turbid and contain flakes of lymph, according to the stage at which death takes place. If the dose is large (several loopfuls of an eighteen hours' agar culture), organisms are found in considerable numbers in the blood and also in the small intestine, but with smaller doses (one-half to one-tenth of a loopful), they are practically confined to the peritoneum, or the cavity may be sterile *post mortem*.

The cholera vibrio is markedly 'enterotropic', and by the intravenous injection of cultures in rabbits, a pathological picture resembling that in human cholera can be produced (Mackie). The animals die in twenty-four to forty-eight hours, and before death there is marked diarrhoea; at autopsy the small intestine is markedly distended with a milky mucous fluid containing whitish flakes of desquamated epithelium and resembling the 'rice-water' stool of cholera. Large numbers of vibrios are present in the intestine, and the gall-bladder is also heavily infected. It must be noted, however, that other vibrios produce a similar effect, e.g. the so-called *paracholera vibrios*.

From the above account it appears that the evidence obtained from experiments on animals on the whole supports the specific pathological relationship of the organism, especially when it is borne in mind that animals do not in natural conditions suffer from the disease.

Experiments on the human subject and accidental infections. Experiments have been performed on the human subject, and accidental infections have also occurred in laboratory workers. In the course of Koch's earlier work, one of the workers in his laboratory was seized with severe choleraic symptoms. The stools were found to contain cholera vibrios in very large numbers. Recovery, however, took place. In this case there was no other possible source of infection than the cultures with which the person had been working. A similar accidental infection from swallowing material from an experimental animal proved fatal to Dr. Oergel. A considerable number of experiments have been performed, which certainly show that in some cases more or less severe choleraic symptoms may follow ingestion of pure cultures, whilst in others no effects may result. The former was the case, for example, with Pettenkofer and Emmerich, who made experiments on themselves, the latter especially becoming seriously ill. In the case of both, diarrhoea was well marked, and numerous vibrios were present in the stools, though toxic symptoms were not pronounced. Metchnikoff also, by experiments on himself and others, obtained results which convinced him of the specific relation of the cholera vibrio to the disease.

As the result of observations on cholera epidemics and of cholera carriers, it may be concluded that only a certain proportion of people are extremely susceptible to cholera.

Toxins. The general statement may be made that filtered cholera cultures as a rule have little toxic action—that is, typical exotoxin is not produced by the organism. It was, however, shown by Pfeiffer that the dead organisms were highly toxic, and that they produced, on injection into guinea-pigs, the same phenomena as living cultures, profound collapse with subnormal temperature being a prominent feature. Pfeiffer considered that the toxic substances are contained in the bodies of the organisms—that is,

they are *endotoxins*—and that they are only set free by the disintegration of the latter. He showed also that when an animal is inoculated intraperitoneally with the cholera organism, and then some time later anti-cholera serum, which produces bacteriolysis, is injected, rapid collapse with a fatal result may ensue, apparently due to the liberation of the endotoxins. Dead cultures administered by the mouth produced no effect unless the intestinal epithelium was injured, in which case poisoning might result. Pfeiffer found that the toxic substances were to a great extent destroyed at 60°C , but even after heating at 100°C . a small proportion of toxin remained, which had the same action. Later, Macfadyen observed that the product obtained by grinding up the organisms frozen by means of liquid air, had a very high degree of toxicity when injected intravenously. Like Pfeiffer, he found that the toxin was in great part destroyed at 60°C . On the other hand, certain observers (Petri and others) have obtained toxic bodies from *filtered cultures*. Metchnikoff, Roux and Taurelli-Salimbeni demonstrated the formation of thermostable diffusible toxic bodies by highly virulent organisms in fluid media. The diversity in the results obtained by various workers seems only explicable on the assumption that different strains vary greatly as regards the diffusibility of their toxic products. It is doubtful whether a true exotoxin is produced and it should be noted that rapid autolysis of the vibrios in culture with the liberation of endotoxin might explain some of the above-mentioned findings. Attempts to isolate the endotoxin by chemical methods have met with some success, it is closely associated with phospholipoid (see Burrows *et al*). antigenic properties seem to be lacking.

Immunity. The guinea-pig or any other animal may be easily immunised against the cholera organism by repeated injections of non-fatal doses of dead vibrios, later, the living organisms may be used. In this way a high degree of immunity against the organism is developed, and further, the blood serum of an animal thus immunised has markedly protective power when injected, *e.g.* intraperitoneally, even in a small quantity, into a guinea-pig along with multiples of the fatal dose of the living organism. Under these circumstances the vibrios undergo a granular transformation and, ultimately, solution. This phenomenon, which is highly specific, is generally known as Pfeiffer's reaction, and was applied by him to distinguish the cholera vibrio from organisms resembling it. The following are the details.

Pfeiffer's reaction. A loopful of an eighteen-hours' virulent agar culture of the organism to be tested is added to 1 c.c. of ordinary broth containing 0.001 c.c. of potent anti-cholera serum. The mixture is then injected into the peritoneal cavity of a young guinea-pig (about 200 grms. in weight), and the peritoneal fluid of this animal (conveniently obtained by means of a 1 c.c. syringe) is examined microscopically after a few minutes and again after twenty, forty, and sixty minutes. If the organisms injected are cholera vibrios, it will be found that they become motionless, swell up into globules, and ultimately break down and disappear—*positive result*. If they are found active and motile then the possibility of their being true cholera organisms may be excluded—*negative result*. In the former case (positive result) there is, however, still the possibility that the organism has been destroyed by the normal peritoneal fluid. A control experiment should be made with 0.01 c.c. of normal serum in place of the anti-cholera serum. If no alteration of the organism occurs with its use, then the conclusion is that a true reaction has been given—the control animal will die from the infection in about twenty-four hours. Corresponding bacteriolytic effects may be obtained by *in vitro* methods, introduced since Pfeiffer's original method.

The serum of an animal immunised by the above method has also marked agglutinating and other antibacterial properties against the cholera vibrio, and these properties closely correspond with Pfeiffer's reaction as regards specificity. For the serological identification of *V. cholera* the agglutination reaction is now used as in the case of other organisms (*vide infra*).

An anti-cholera serum has little protective effect against the toxic action of the dead vibrios, and Pfeiffer maintained that little or no antitoxin to the endotoxin can be produced. On the other hand, Macfadyen, by injecting the endotoxin derived from the vibrios by grinding, obtained a serum which had antitoxic as well as agglutinating and bacteriolytic properties. Metchnikoff and others also obtained antitoxic sera for the extracellular toxins which they prepared.

For therapeutic purposes several antisera which are supposed to have antibacterial have been used. They are of doubtful specific and bactericidal character, but it cannot be denied that they possess antitoxic properties. The possession of marked antitoxic properties by these sera has not been established experimentally.

The serum of cholera convalescents has been found to possess protective and increased bactericidal action. These properties of the serum may be present eight or ten days after the attack of the disease, but are most marked four weeks after; they then gradually diminish. Specific agglutinins appear in the serum of cholera patients, as in other diseases. They are most marked in convalescence, reaching the maximum in from two to three weeks from the onset of the disease, the serum then agglutinating in a dilution of 1 in 400 or even 1 in 1,000 (Greig). Agglutinins are also often present in the blood of carriers. It should be noted, however, that normal serum may sometimes have an agglutinating effect on the cholera organism in dilutions up to 1 in 20.

Anti-Cholera Vaccination. Preventive inoculation against cholera first attracted attention largely as the result of Haffkine's work in India. On the analogy of Pasteur's anti-anthrax inoculation, injections of attenuated organisms were administered first of all, and were followed by the injection of cultures of organisms of exalted virulence which had been passed repeatedly through the peritoneal cavity of guinea-pigs. Killed suspensions of the organism prepared from cultures on agar have more recently been extensively used as vaccines. They are prepared according to the general technique described in the Appendix. Adequate dosage of the vaccine is essential—two injections of 5,000 and 10,000 million organisms at an interval of a week. The effect of the preventive inoculation of troops exposed to cholera infection in time of war, both in reducing the incidence and lessening the mortality of the disease, has been illustrated (see Greenwood and Yule). The results of field tests of anticholera vaccination in India have been reported by Russell along with careful statistical analyses of records of cholera attacks and deaths among immunised persons and controls. Both oral and subcutaneous methods of administration were investigated, Besredka's cholera bilvaccine being used in the former case. Russell has concluded that a high degree of immunity is conferred by both methods but that the effect of subcutaneous vaccination is superior to that of the orally administered vaccine. (See also Adiseshan *et al.*; Chandra Sekar.)

Allied Vibrios. *El Tor vibrio*. In 1905 Gotschlich obtained six different strains of a vibrio which conformed in cultural and serological reactions to *V. cholerae*, but differed from the classical cholera organism in having marked hæmolytic action (as already described), and also in producing a rapidly acting extracellular toxin. The organisms were obtained at El Tor from the intestines of pilgrims who had died with dysenteric symptoms; and there were no cases of cholera in the vicinity. There has been some difference of opinion as to whether these organisms are to be regarded as a distinct species or as variants of the cholera vibrio and whether the hæmolytic property represents a fundamental difference from *V. cholerae*. The antigenic relationships of the El Tor vibrio to *V. cholerae* will be considered later, but it may be said that while the former may be found occasionally in choleraic cases and in healthy carriers, it is never associated with typical

epidemic cholera. According to Takita the El Tor vibrio produces a true exotoxin which is thermolabile and is neutralised by antiserum. It has been stated that this type of vibrio differs also from *V. cholerae* in giving the Voges-Proskauer reaction.

Paracholera vibrios Certain observations have shown that there occur groups of cases with choleraic symptoms or merely diarrhoea, in which the vibrios present differ in some respects from the cholera organism. Such cases

in cases of the disease, similar organisms have been obtained from the stools of contacts—that is, carriers occur. In those affected the vibrios are often present in the stools in large numbers, and on isolation are found to have the morphological and cultural characters of the cholera organism, they are also virulent to the guinea-pig on intraperitoneal injection, and to the rabbit on intravenous injection, as in the case of the classical *V. cholerae*. They are, however, markedly hæmolytic, when tested both on blood agar plates and with suspensions of sheep's red corpuscles. Further, they differ serologically from the cholera organism even after repeated subculture—when tested in the form of suspensions of agar cultures they are not agglutinated at 37° C by an anti-cholera serum, and they react negatively in Pfeiffer's reaction. Antisera for these organisms do not agglutinate the typical cholera vibrio. They also differ serologically amongst themselves, and several varieties may be distinguished in this way (Mackie).

Similar organisms have been isolated from water supplies in the East

most highly parasitic type. Some of these vibrios may be purely saprophytic, occurring in water supplies in certain regions of the world, or establishing themselves as commensals in the bowel. Others may occupy an intermediate position and possess potentially pathogenic properties, e.g. the *paracholera vibrios*.

In addition to the *paracholera* organisms described above, other vibrio types have also been isolated from choleraic cases, e.g. *V. metchnikovi*, *V. proteus*, phosphorescent vibrios, etc. No definite statement can be made as to the pathological relationships of these types in the human subject.

Taylor, Pandit and Read have reported the results of an investigation in India of a large cholera serum

healthy persons, logically and biochemically. Thus, thirty-one different serological types were recognised. Biochemically they were classified according to Heiberg's criteria (fermentation of mannose, saccharose, and arabinose), along with the cholera-red and Voges-Proskauer reactions. While cholera-red positive and negative inagglutinable strains were generally positive and negative respectively in the Voges-Proskauer test, *V. cholerae* was found to be negative in the latter reaction. These workers have concluded that in India generally no vibrio of serological characters other than the classical is responsible for epidemics of cholera.

Antigenic Structure of the Cholera Vibrio and Allied Organisms. As pointed out above, strains of the cholera vibrio isolated from the typical epidemic disease usually show a high degree of specificity in their agglutination

reactions when tested by the ordinary methods, and in this way they have been clearly distinguished from 'cholera-like' and paracholera vibrios. The El Tor vibrio has usually been found to behave like the true cholera vibrio in serological reactions though differing from it in being actively haemolytic. The agglutination reactions of these organisms have in the past been carried out with saline suspensions of agar cultures, incubation being at 37° C. for a short period. Douglas, using formalised suspensions and incubating at 50° to 55° C., elicited a group effect between *V. cholera* and a paracholera vibrio. The different results obtained by the two methods were later emphasised by Mackie who showed that the essential serological distinction between *V. cholera* and paracholera vibrios was more clearly demonstrated by the former technique. The cholera vibrio, like other flagellate bacteria, possesses antigenic constituents associated respectively with the flagellum and body of the organism (Balteanu). From the work of Shousha, Abdoosh, Gohar, and others it appears that the classical cholera vibrio contains constant H and O antigens, the latter being multiple. But while the same H antigen occurs in other vibrios the O antigen is specific. This work was extended by Gardner and Venkatraman, who suggested a classification of vibrios according to their H and O antigens. They recognise a 'cholera group' of biochemically similar vibrios possessing a common H antigen and they divide this into subgroups each with a specific O constituent. I, comprising the classical *V. cholera* and the El Tor vibrio; II, III, etc. which are mostly haemolytic and include the various races of paracholera vibrios, 'cholera-like' vibrios, and also some strains classified as El Tor vibrios. Within subgroup I serological types have also been recognised and can be differentiated by agglutinin-absorption tests with O antisera, the difference depending on subsidiary O constituents. Two such types have been designated by the names of standard strains, 'Inaba' and 'Ogawa'. The same form of type differentiation can be demonstrated among El Tor vibrios.

The antigenic constitution of the vibrios of the cholera group is of considerable complexity and in addition to specific O factors a non-specific component is also demonstrable. Further, according to White, the transformation to the rough (R) form is associated with a loss of the specific O antigen, and the non-specific O component then becomes as it were unmasked. White has also described variants (designated p) which have lost their 'rough' O antigen with the unmasking of another common constituent. The chemical nature of these components has been studied by Linton and his co-workers in India. They find that specificity depends on protein and carbohydrate substances and they have not formed a separate classification based on chemical constitution. Linton and his co-workers have shown that a cholera strain may yield a derivative whose serological character is different from the parent strain in virtue of changes in its chemical structure. The antigenic and chemical inter-relationship and differences among these organisms require further study (see Linton, 1910).

From the point of view of identifying the epidemiologically important classical *V. cholera*, it may be said that in the case of an organism with the characteristic morphological and biochemical characters agglutination to titre by an anti-O serum for sub-group I is conclusive evidence of its nature.

It has been noted that along with *V. cholera* there may occur in the stools of cholera patients a vibrio which agglutinates with anti-cholera serum (Crendropoulos; Greig). This vibrio has not been finally settled, though under certain conditions, may undergo such

alteration as to become inagglutinable by anti-cholera serum, e.g. in the bowel of convalescent patients and in water (Tomb and Maitra). Recent observations on antigenic variation may offer some explanation of these findings. It has been pointed out by Doorenbos that under the action of a bacteriophage, *V. cholerae* may become inagglutinable and also acquire hæmolytic properties. It must also be mentioned that strains when first isolated may be inagglutinable, but after repeated culture react to an anti-cholera serum.

Cholera Bacteriophage. A bacteriophage which is active towards the cholera vibrio has been obtained by various workers, and a method for its isolation from the stools of cholera cases was first described by d'Herelle. D'Herelle and Malone endeavoured to correlate the activity of this bacteriophage in the intestine of cases with recovery from the disease, and stated that in India outbreaks of cholera can be controlled by the addition of bacteriophage to water supplies. Bacteriophage preparations were also applied in the treatment of cholera. D'Herelle and his co-workers claimed that if the bacteriophage is administered sufficiently early in the illness a considerable reduction in mortality results.

The study of the cholera bacteriophage and its practical application in prevention and treatment was extended by Asheshov and by Morison. Asheshov pointed out the existence of different types of phage each producing different forms of 'clearings' or 'plaques' on cultures of the vibrio and by repeated isolation from individual plaques succeeded in separating 'pure line' strains. Three types (A, B and C) were first recog-

dary growth, whereas the several types when used in combination tended to prevent this. Thus the principle was adopted in using phage for the treatment of cholera to combine all available types acting on the vibrio and to incorporate in a preponderating proportion one which acted quickly, the others serving to prevent secondary growth. Morison demonstrated nine different types of cholera phage and showed that by combining different types complete lysis of a cholera culture is produced without any subsequent secondary growth. He applied practically such combined phages for the control of cholera in Assam, the material being distributed on a large scale and taken by mouth. Prophylactic and therapeutic effects have been claimed, as well as control of the spread of the disease.

Bacteriological Diagnosis. In the first place, the stools may be examined microscopically. Dried film preparations should be made and stained by any ordinary stain, though carbol-fuchsin diluted four times with water is specially to be recommended. Hanging-drop preparations should also be made, by which method the motility of the organism can be readily seen. By microscopic examination the presence of vibrios will be ascertained, and an idea as to their number obtained. In some cases the cholera vibrios are so numerous in the stools that a picture is presented which is obtained in no other condition, and a microscopic examination may be sufficient for immediate purposes.

If the organisms are very numerous, plates of Dieudonné's medium may be inoculated at once and a pure culture obtained from one of the colonies. Aronson's medium has been used as an alternative.

If the vibrios occur in comparatively small numbers, the best method is to inoculate peptone solution (1 per cent) standardised to pH 8.0, and incubate for six to eight hours. At the end of that time the vibrios will be found on microscopic examination in large numbers in the surface pellicle, and thereafter plate cultures can readily be made on Dieudonné's medium. If the vibrios are very few in number, the peptone solution which has been inoculated should be examined at intervals till vibrios are found microscopically. A second tube of peptone solution should then be inoculated and subcultures made later on Dieudonné plates. Though Dieudonné's medium in virtue of its alkalinity restrains the growth of most other intestinal bacteria and thus yields, as a rule, a practically pure growth of *V. cholera*, in all cases the purity of the strain must be ensured by isolating from single colonies.

Preserving medium for the transmission of stools intended for isolation of V. cholera
A stock solution is made up of 10.405 grms. H_2PO_4 and 14.019 grms. NaCl in distilled solution

grms. dried sea-salt are dissolved in the fluid (sea-salt may be replaced by 20 grms of the following mixture— NaCl 27 grms., KCl 1 grm., $\text{MgCl} \cdot 6\text{H}_2\text{O}$ 3 grms., and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.75 grms.), the solution is filtered, distributed in 10 c.c. amounts in screw-capped bottles and sterilised in the autoclave. The pH should be 9.2. Of a specimen of stool 1 to 3 grms. according to consistency are emulsified in a bottle of preserving medium.

When a vibrio has been obtained in pure condition by the above methods it should be tested, as regards agglutination, with a high-titre anti-cholera serum prepared by immunising rabbits with a suspension of an agar culture of classical *V. cholera* which has been previously boiled for two hours. At the same time the cultural characters and hæmolytic property should be tested. The characters according to which a vibrio strain may be regarded as a true *V. cholera* have been detailed earlier in the chapter. In summary it can be said that vibrios which fail to give the following reactions can be excluded—fermentation with acid reaction (without gas) of glucose, saccharose, and mannose; arabinose not fermented; cholera-red reaction positive; Voges-Proskauer reaction negative; non-hæmolytic for sheep red cells; agglutination by an O-specific anti-cholera serum.

It must be remembered, however, that failure to isolate classical *V. cholera* may be experienced in a proportion of clinically declared cases even in time of epidemic. If the strain reacts negatively with anti-cholera serum it may be one of the paracholera group, and similar tests should be made. At post-mortem examination of a suspected case portions of the unopened bowel some inches long at the middle of the ileum and just above the ileo-cæcal valve should be ligatured off and transmitted for bacteriological examination, immersed if necessary in sterile saline.

OTHER VIBRIOS

The cholera vibrio belongs to a group of organisms which resemble it in microscopic and cultural characters. Some of these have been found to produce disease in animals, e.g. *V. metchnikovi* originally isolated from an epidemic disease of fowls, some again have been recovered from water or materials suspected of harbouring the cholera organism, e.g. Finkler and Prior's vibrio (*V. proteus*) obtained from decomposing feces from a case of *cholera nostras*; while others have been derived from various sources, e.g. Deneke's vibrio (*V. tyrogenus*) recovered from cheese, Miller's vibrio isolated from a carious tooth and probably the same as *V. proteus*. Before the importance of serological tests for the identification of *V. cholera* was established, various cultural and biological characters were relied on for distinguishing them. Thus while Metchnikoff's vibrio closely resembles the cholera organism both in cultural appearances and in giving the cholera-red reaction, it can be readily distinguished from the latter by the effects of inoculation in animals, especially in pigeons and guinea-pigs. Intramuscular inoculation of small quantities of pure culture in pigeons is followed by septicaemia, which produces a fatal result usually within twenty-four hours. Inoculation with the same quantity of the cholera vibrio produces practically no result, even with large quantities, death is rarely produced. Metchnikoff's vibrio produces somewhat similar effects in the guinea-pig to those in the pigeon, subcutaneous inoculation being followed by extensive hæmorrhagic œdema and a rapidly fatal septicaemia. Young fowls can be infected

by feeding with virulent cultures. Organisms which are apparently the same as the *Vibrio metchnikovi* have been cultivated from water and from choleraic cases. Finkler and Prior's (Fig. 104) and Deneke's organisms liquefy gelatin rapidly and do not give the cholera-red reaction. Certain vibrios isolated from water are phosphorescent in cultures growing at 22° C (*V. phosphorescens*). Phosphorescent vibrios have also been found in the stools of cholera-like cases. None of these organisms is agglutinated by an anti-cholera serum. Anaerobic vibrios have been isolated from the mouth and upper respiratory passages.

Vibrio foetus. This organism was described by Theobald Smith as the causal organism of a form of infectious abortion of cattle. It has also been found in abortion of sheep. The organism can be isolated from the placenta and the foetus. It is somewhat variable in morphology, and may appear either as a typical vibrio about 2 μ in length or as a spirillum with 2 to 4 complete coils. It is motile and has a flagellum at one or both ends. The staining reaction is Gram-negative. In cultural characters it is micro-aerophilic, and when first isolated can only be cultivated with difficulty. The addition of a few drops of defibrinated horse blood to the condensation water of an agar slope permits growth in the fluid. After repeated subculture a delicate surface growth may be obtained. The organism does not exhibit any fermentative properties. Laboratory animals are not susceptible to experimental inoculation.

Vibrio jejuni has been found associated with a diarrhoeal condition of cows in America. It resembles *V. fetus* and is biologically related to it.



FIG. 104. Finkler and Prior's vibrio, from a twenty-four hours' agar culture. Stained with dilute carbol-fuchsin. $\times 1,000$

specimen stained with methylene blue, the fully developed spores are uncoloured except at the periphery, so that the appearance of a small ring is produced; if a powerful stain such as carbol-fuchsin be applied for some time, the spores become deeply coloured black.



FIG. 109 Stab culture of the tetanus bacillus in glucose gelatin, showing the lateral offshoots (after Kitasato) Natural size

the growth over the surface of the medium (Non-motile variants may yield well delimited colonies without these branching projections.) In *fluid media* under anaerobic conditions there is at first a slight turbidity, and later a thin layer of a powdery deposit on the walls of the vessel. *Meat medium* undergoes little change in colour, but there may be slight digestion and discoloration of the meat. *Coagulated serum* is softened but not liquefied. On *blood agar* hæmolytic zones develop round the colonies. None of the sugars commonly used are fermented. All the cultures have a peculiar odour of burnt organic matter. In making subcultures in fluid media a considerable amount of the original growth should be used for the inoculation.

...many to be found, the latter having no distinctive features.

CULTURAL AND BIOCHEMICAL CHARACTERS. In the case of the ordinary media growth takes place

...of copper, it is only necessary to heat it at 100°C . to expel dissolved oxygen and then on inoculation an abundant growth of the bacillus results. In cooked-meat medium the tetanus bacillus, like other anaerobes, will grow without exclusion of air; this is due to the reducing action of the particles of minced meat. It grows best at 37°C . The minimum growth temperature is about 11°C ., and below 22°C growth takes place very slowly. In *glucose gelatin stab culture* (in which growth is often difficult to obtain) there commences, an inch or so below the surface, a growth consisting of fine straight threads radiating out from the wire track (Fig. 109), and rather longer in the lower than in the upper parts of the tube. Slow liquefaction of the gelatin takes place, with slight gas formation. In *agar stab culture* the growth is somewhat similar, there being a slightly nodular line of growth along the wire track, with irregular short offshoots passing out into the

medium (Fig. 119, A) and slight formation of gas. On anaerobic *agar plates* colonies have a feathery outline (Fig. 110) and consist mainly of long branching processes which tend to spread



FIG. 110 Colonies of the tetanus bacillus on an anaerobic agar plate, seven days old $\times 50$

Isolation of pure cultures (1) Advantage may be taken of the resistance of the spores to heat. A tube of cooked-meat medium is inoculated, and incubated at 37°C for forty-eight hours, at the end of which time spore-bearing bacilli can often be observed microscopically. The culture is then kept at 70°C for from three-quarters to

one hour, with a view to killing all organisms except spores. From such material anaerobic plate cultures are prepared by the methods described in the Appendix. This method of isolation is frequently unsuccessful, because along with the tetanus bacilli, both in its natural habitats outside the body and in the pus of wounds, other spore-forming obligatory and facultative anaerobes occur, which grow faster than the tetanus bacillus, and thus overgrow it.

(2) A method recommended by Fildes depends upon the fact that under suitable conditions *B. tetani* grows as a spreading film on solid medium and extends beyond the growth of other organisms. With material from a case of tetanus it is advisable to enrich the growth first by inoculating a tube of freshly boiled blood broth, which is incubated anaerobically at 37° C for two to four days. Then from this culture the condensation water of a sloped tube of digest blood agar is inoculated and the tube is incubated anaerobically at 37° C. After twenty-four to forty-eight hours the extreme edge of the film of growth, seen with a hand-lens as a tangle of extremely fine filaments, is found on microscopic examination to consist of a culture of the tetanus bacillus; subcultures from the margin yield pure growths of the organism. The detection of the edge of the growth is facilitated by using agar tubes which have been kept until the medium has become slightly dry at the top. The only organism which is likely to spread as extensively as *B. tetani* is *B. proteus*. The latter can be eliminated by heating the mixed growth containing spores of *B. tetani*.

VIABILITY. The organism possesses a very high degree of resistance to physical and chemical agents in virtue of its spores. These often withstand boiling for forty to sixty minutes, but in some cases they may not survive for ten minutes. They can be kept in a dry condition for many months without being killed or losing their virulence. They have also high powers of resistance to many antiseptics; for example, they withstand 5 per cent phenol and 1 in 1,000 mercuric chloride for two weeks or even longer. On the other hand, a 1 per cent watery iodine solution or hydrogen peroxide (10 volumes) destroys them within a few hours.

Occurrence in Nature. By inoculation of animals (*vide infra*), *B. tetani* has been proved to be frequent in richly manured soil, e.g. garden earth; it also occurs in street dust. It flourishes in the intestines of many—especially herbivorous—animals and such carriers are responsible for distributing the spores in nature. Kerrin has demonstrated the organism in a variety of animals: rat, dog, rabbit, guinea-pig, mouse, horse, cow, sheep, pig. It has also been recovered in some cases from the intestine of man. In soil the organism occurs in the form of spores, and it is doubtful whether the tetanus bacillus can exist as a saprophyte. In turn spores from the soil are ingested by animals and germinate in the intestine. It is not definitely known whether the carrier state in animals is transient or permanent.

Relationship to Disease and Pathogenic Effects. The proof that the tetanus bacillus is the cause of the disease is complete, since pure cultures reproduce all the characteristic effects. Failure to isolate it from some cases of the disease is very probably due to the small numbers in which it sometimes occurs. The tetanus bacillus by itself lacks the power of invading the tissues. It obtains a foothold only under favouring conditions such as are afforded by a mixed infection, the presence of foreign matter or necrotic tissue. That this is the case first appeared from experiments of Vaillard and others in which the spores freed from toxin by heating at 80° C were injected. The animals remained healthy, but the spores persisted in a living state for a considerable time, and tetanus was set up in guinea-pigs when after some weeks, staphylococci were injected subcutaneously into the same site as the spores. Similarly in rabbits after intravenous injection of such detoxicated spores a simple fracture of the femur caused the disease to develop. Vaillard and his co-workers also noted that *B. prodigiosus*, various aerobic soil organisms, and mixtures of pyogenic organisms injected along with *B. tetani* spores led to the development of tetanus. The injection of

sterile soil favours the germination of these spores in the tissues, and Bullock and Cramer have attributed this effect to the ionisable calcium salts in the soil. Various chemical substances (such as those capable of damaging or devitalising the tissues) have also been found to promote tetanus when injected along with spores, e.g. quinine, lactic acid. According to Tulloch, the toxin of *B. welchii* exerts a marked effect in predisposing to tetanus when *B. tetani* spores are injected. The factors that promote the development of *B. tetani* in the tissues have been studied, by Mackie, McLachlan and Anderson, who found that certain animals, e.g. mice, can resist massive doses of spores *per se*; small doses, however, when injected along with sublethal doses of *B. welchii*, *B. adematensis*, *B. coli*, or *Staphylococcus aureus*, frequently produced tetanus in these animals. Exposure to cold also precipitated tetanus in animals even a considerable time after injection of the spores. Vaillard and his co-workers attributed the non-pathogenicity of *B. tetani* spores to their being rapidly phagocytosed in the healthy tissues, and assumed that those factors which interfered with this protective mechanism, e.g. pyogenic infection, would favour their development in the body. Fildes has claimed that the failure of the spores to germinate is due to the unfavourable oxygen tension of the healthy tissues, and that germination, with resultant tetanus, is dependent on factors producing tissue asphyxia. The inhibitory and adjuvant factors concerned are probably complex, and depend not only on phagocytosis and the oxygen tension of the tissues, but also on the parasitic properties of the organism and the various defences of the tissues. In experiments on tetanus following vaccination Armstrong found that when the site of inoculation with a mixture of vaccinia virus and *B. tetani* spores was kept covered throughout by an impermeable dressing, the majority of the monkeys and rabbits so treated developed tetanus, but when the inoculated area was freely exposed to the air after the first eighteen hours, the occurrence of tetanus was exceptional. There may be a very long period of latency of spores. Thus the organism has been cultivated from the tissue of an abdominal scar of a woman who had suffered ten years previously from tetanus following a gynaecological operation (Bonney *et al.*).

Tetanus bacilli tend to remain localised at the site of entry in a wound, and the toxin which they produce is absorbed and acts on the central nervous system.

(a) *The disease as arising naturally.* The spasms early affect the extensor muscles of the trunk (descending tetanus). There is in most cases a definite wound, often of a ragged character, which has either been made by an object soiled with earth or excretal matter, or which has become contaminated with these substances and which consequently shows pyogenic infection. For example, tetanus has followed wounds received from toy pistols loaded with cartridges having contaminated wads. It is important to note that in some cases the wound through which infection has taken place may be very small, in fact, may consist of a mere abrasion; especially in the tropics, it may possibly be merely the bite of an insect. In some parts of the world infection through the umbilicus originates a high mortality from the disease in newly-born infants. The absence in many cases of an obvious channel of infection has given rise to the term 'idiopathic' tetanus. In tetanus following clean operation wounds, imperfectly sterilised catgut ligatures have sometimes been the source of infection. Surgical catgut being prepared from the submucous layer of sheep's intestine, the raw material is liable to contamination with the spores of *B. tetani* and other intestinal anaerobes, e.g. *B. welchii*, and as a result of the spinning process these spores may come to be embedded in

the ligature. The resistance of *B. tetani* spores to various physical and chemical agencies has been mentioned above, and in the past imperfectly sterilised catgut ligatures have not infrequently been used for surgical purposes¹

During the war of 1914-18 tetanus in the wounded assumed a certain degree of prevalence, but the clinical type was modified in consequence of the wide application of the prophylactic injection of anti-tetanic serum (*vide infra*). In the first place there was a tendency to the prolongation of the incubation period, instances where this was extended to many months being not uncommon. In such cases there was usually an unhealed septic wound, often containing foreign bodies, and the attack of tetanus was sometimes precipitated by operative procedures, in some cases the wound had healed and tetanus followed operation for the removal of foreign bodies in the tissues. Again the disease tended to assume the type seen in some animals, the muscles in the neighbourhood of the wound being first affected, local hardness and stiffness, pain, and exaggeration of local reflexes were often the first, and sometimes the only, clinical phenomena. Such cases of tetanus were also apparently more amenable to treatment with anti-tetanic serum. In the recent war the incidence of tetanus in British troops was remarkably low. Active immunisation with tetanus toxoid (*vide infra*) and the prophylactic use of antitoxin contributed to this result.

The pathological changes found *post mortem* are not striking. There may be hæmorrhages in the muscles which have been the subject of the spasms. These are probably due to mechanical causes. In the nervous system the most noticeable feature is the occurrence of irregular patches of slight congestion which are not limited particularly to grey or white matter, nor to any tract of the latter. These patches are usually most marked in the grey matter of the medulla and pons. Microscopically there is little of a definite nature to be found. There is congestion, and sometimes minute hæmorrhages. The ganglion cells may show appearances which have been regarded as degenerative in nature, and similar changes have been described in the white matter. The only marked feature is thus a vascular disturbance in the central nervous system, with a possible tendency to degeneration in its specialised cells. Both of these conditions are probably due to the action of the toxin of the bacillus. In the other organs of the body there are no constant changes.

(b) *The artificially-produced disease.* The disease can be communicated to animals by any of the usual methods of inoculation, but does not arise from feeding with the bacilli, whether these contain spores or not. In mice, after inoculation with a small dose of a broth culture, symptoms appear in a day, and death occurs in two or three days. Guinea-pigs and rabbits require larger doses, and death does not occur so rapidly. Usually in small animals injected subcutaneously in a limb the spasms begin in that limb near the point of inoculation and this is the rule when the inoculation is intramuscular (local tetanus). Later the condition extends to other muscles and may become generalised (ascending tetanus). In the case of inoculation intravenously or into a non-muscular region, the spasms begin in the extensor muscles of the trunk, as in the natural disease in man (descending tetanus). After death there is found, at the seat of inoculation, slight hyperæmia without pus formation. The bacilli diminish in number, and may be absent at the time of death. The organs generally show little change.

Kitasato stated that in his earlier experiments the quantity of culture medium injected along with the bacilli already contained enough of the toxin

¹ The sterility of surgical catgut is now controlled in this country under the provisions of the Therapeutic Substances Act.

formed by the bacilli to cause death. The symptoms came on sooner than by the injection of spores freed from toxin, and were, therefore, due to the toxin already present. In his subsequent work, he employed splinters of wood soaked in cultures in which spores were present, and subsequently subjected for one hour to a temperature of 80°C . The latter treatment not only killed all the vegetative forms of the organism, but was sufficient to destroy the activity of the toxin. When such splinters were introduced subcutaneously, death resulted from the development of the spores which they carried. In this way he completed the proof that the bacilli by themselves can form toxin in the body and produce the disease.

Toxin. The toxic properties of bacterium-free filtrates of pure cultures of the *B. tetani* were investigated in 1891 by Kitasato. When the filtrate, in suitable doses, was injected subcutaneously into mice, tetanic spasms developed first in muscles contiguous to the site of inoculation and later all over the body. Death resulted. He found that guinea-pigs were more susceptible than mice, and rabbits less so. The toxin is easily injured by heat; exposure for a few minutes at 65°C destroys it; it is also destroyed by twenty minutes' exposure at 60°C , and by one and a half hours' at 55°C . It is destroyed by various chemicals such as pyrogallol and acids, and also by sunlight. Toxin is very stable when dried.

To prepare the toxin, freshly made veal broth not too long autoclaved should be used, and a massive inoculation made, preferably from a fluid culture. Individual strains of the bacillus differ in their capacity for producing toxin. The culture must be incubated under anaerobic conditions, and the maximum toxicity is developed in from five to fifteen days. Behring pointed out that after the filtration of cultures containing toxin, the latter may very rapidly lose its power, and in a few days may only possess 1/100th of its original toxicity. This is due to such factors as temperature and light, and especially to the action of oxygen. When dilutions of toxin are to be prepared, broth should be used and not saline which leads to rapid deterioration. Toxin may be preserved by precipitation with ammonium sulphate, the precipitate being dried and kept as a powder. The effect of certain agents, e.g. formaldehyde, on the crude toxin is to form a *toxoid*, similar to that produced in the case of diphtheria toxin, and it is also true here that the toxoid while losing its toxicity may still retain the power of producing immunity against the potent toxin. Further, it has been shown that such crude toxin may be used as an adjuvant of toxic substances. Ehrlich found that beside

there are certain substances which are capable of destroying the activity of red blood corpuscles of certain species. This hæmolytic action is called *tetanolysin*. It does not occur in all samples of crude tetanus toxin, nor is it found when a broth culture of the bacillus is filtered through porcelain. Tetanolysin also has the power of originating an antitoxin, so that certain anti-tetanic sera can protect red blood corpuscles against its action; this protective effect can occur even when the corpuscles have already combined with the lysin. Madsen, studying the interactions of this anti-tetanolysin with the tetanolysin, showed that phenomena can be demonstrated similar to those noted by Ehrlich as occurring with diphtheria toxin, which the latter interpreted as indicating the presence of degenerated toxin (toxoid) in the crude poison. A leucocidin has also been observed in culture-filtrates. It is important to note that the toxin may not be found in mixed cultures of *B. tetani* along with other organisms (Tulloch). Special problems are involved in the production of a very active toxin necessary for conversion into toxoid for use in immunisation. Mueller and Miller have obtained such a product

by growing a highly toxigenic strain of *B. tetani* in a medium containing a mixture of ox heart extract and autolysed pig's stomach.

The toxin is one of the most powerful poisons known. Even with a crude ammonium sulphate precipitate from cultures in broth the fatal dose for a mouse is often 0.0001 of a milligram. If the susceptibility of man be the same as that of a mouse, the fatal dose for an average adult would be about 0.5 of a milligram. Animals differ very much in their susceptibilities to the action of tetanus toxin. According to Fildes' computation, if the M.L.D. per gram weight for a horse be taken as unity, that for the guinea-pig would be 2 times the amount, the mouse 4, the goat 6, the rabbit 24, the dog about 300, the cat 2,400, and, according to v. Lingelsheim, the goose, 12,000, the pigeon 48,000, and the hen 360,000. These figures, however, may be affected by various factors such as the individuality of the animals, their age (the younger being more sensitive), etc. Also, when different specimens of toxin are tested in parallel in the rabbit and the mouse, it is found that the ratio of the lethal doses per unit weight in the two species may vary 150-fold, thus suggests strongly that toxic filtrates contain multiple lethal factors (see Smith). While in a number of animal species the lethal dose of toxin is practically the same on intravenous, intramuscular, or subcutaneous administration (van Genderen, Abel), on the other hand, different species vary in their sensitiveness to intracerebral injection. Thus the fatal dose of toxin for the guinea-pig is not smaller when it is injected intracerebrally than when given subcutaneously. In the rabbit, however, a much smaller dose of tetanus toxin proves fatal when injected into the brain than when given subcutaneously. This probably depends on the relative affinities of different organs for the toxin.

Little is known of the nature of tetanus toxin. Uschinsky found that the tetanus bacillus can produce its toxin when growing in a fluid containing no protein matter. Eaton and Gronau have described a method of purifying the toxin by precipitation with diphtheria toxin. Crude toxin

M.L.D. yielded after purification

While practically all the N in a purified diphtheria toxin is precipitated by trichloroacetic acid, only 8 to 18 per cent. of the N in purified tetanus toxin is so precipitated. Recently Pillemer and associates have obtained a crystalline product which in relation to its nitrogen content, is fully 200 times more toxic than the above. They precipitated Mueller's preparation of toxin with 40 per cent. methyl alcohol at -5°C . and a pH of 5.2, the precipitate, which dissolved in 0.15 M sodium acetate, was purified by repeated treatment in the cold with varying concentrations of alcohol at an acid pH, so that the toxin appeared in the supernatant or the precipitate according to the conditions. Finally the toxin was repeatedly recrystallised from solution by addition of 25 per cent. alcohol at -8°C . and a pH of 5.1. The crystals disintegrate quickly at temperatures above -5°C , they appear to consist solely of protein.

Non-toxic variants. Fildes has shown that from typical toxigenic strains non-toxic variants may be developed, and non-toxic forms identified as tetanus bacilli by serological criteria have been isolated by various workers from different sources, though they are apparently most frequent in soil.

Tetanus toxin is readily absorbed and produces its characteristic effects when injected subcutaneously, intramuscularly, intravenously, intraperitoneally, or intrathecally. But it is not absorbed when placed on the surface of granulation tissue, e.g. in a wound a week old. That the toxin is not destroyed, however, is shown by the fact that if later the granulation tissue is broken

down mechanically, absorption occurs and tetanus develops. In the healthy alimentary tract toxin does not lead to tetanus; this is probably to be accounted for by non-absorption as well as by its destruction. In human cases it has occasionally been demonstrated in the blood and the cerebro-spinal fluid.

A striking feature of the action of tetanus toxin is the occurrence of an incubation period between its introduction into an animal's body and the appearance of symptoms. This varies according to the species of animal employed; on subcutaneous injection in the guinea-pig it is from thirteen to eighteen hours and in the horse five days. Increase of the dose, however, within limits shortens the incubation. In man the interval between the injury and the appearance of tetanic symptoms is usually from two to fourteen days, but this period may be lengthened, and the bacilli or spores may remain a considerable time shut up in a wound before producing effects. The longer the incubation period and the more slowly the reflex muscle spasm becomes generalised, the more favourable the prognosis. In chronic cases spontaneous recovery is not uncommon.

With regard to the action of the toxin, it has been shown that when a minimum lethal dose is introduced directly into the blood stream, it disappears rapidly from the circulation, or when injected subcutaneously it cannot be demonstrated in the blood at all. This, no doubt, is partly due to its passing into the lymph, but mainly to its combining with certain tissues, especially the central nervous system, and the latter has been found to fix the toxin *in vitro*. But when a large amount of the toxin is injected into the body or produced there, it circulates in the blood stream. Accordingly, it is a point of practical importance to ascertain by test that all antisera intended for therapeutic use are free from tetanus toxin. Neglect of this precaution has led to fatalities from the employment of the serum of horses withdrawn during the incubation period of tetanus. The toxin has an effect on the central nervous system which can be clearly demonstrated by injecting it directly into the spinal cord. After an incubation period which is much shorter than when the toxin is injected by any other route, tetanic spasms start in the muscles whose nerve supply corresponds to the site of inoculation and then spread to other muscles as the toxin diffuses in the cord. The spasms are due to the raised excitability to afferent stimuli in the motor cells of the anterior cornua. According to the earlier investigators (Marié and Morax, Meyer and Ransom; Permin; and others), the local muscular spasm is wholly explicable on this basis, the toxin reaching the cord by passing up the motor nerves. Recently by the use of newer methods Acheson and associates have confirmed that toxin injected intramuscularly affects selectively the responses of the related segments of the spinal cord on the same side, even when no local tetanus is produced. The precise mode of spread of toxin in the nerves remains doubtful, however. According to one view, the axis cylinders are the conductors; on the other hand, it has been held that the toxin ascends in the neural lymphatics or tissue spaces, but for these two routes there appears to be no anatomical evidence. According to Abel and others, the local muscular contraction, which is the initial phenomenon in ascending tetanus and which also follows the direct injection of a minute dose of toxin into the substance of a muscle, is due to the local action of the toxin. But the occurrence of local spasm depends in some way on the intact condition of the connection of the muscle with the spinal cord by means of the motor nerve. When the nerve is cut, a subsequent injection of toxin into the corresponding muscle does not produce spasm. Harvey has shown, however, that if toxin is injected into the muscle soon enough

after the nerve is cut, *i.e.* while the motor-nerve endings are still intact, rigidity is produced. On the other hand, when spasm has become fully developed, section of the motor nerve no longer abolishes it. Abel stated also that the injection of a considerable dose of tetanus toxin into the sciatic nerve of a dog is not followed by tetanus, either general or local, provided that leakage of the toxin into the surrounding tissues is prevented. In the light of this work the general muscular spasm of descending tetanus is accounted for by the diffuse action on the central nervous system of toxin conveyed to it by the circulation. Friedemann and his co-workers disagree with the views adopted by Abel. They consider it unlikely that the toxin is transmitted to the central nervous system by the circulation, since after intramuscular injection tetanus is produced in animals having a high concentration of antitoxin in the blood. They have shown that when the toxin is injected in the hind limbs a much larger amount of antitoxin (injected intravenously) is needed to prevent tetanus than when the toxin is introduced intravenously. Moreover, severance of the sciatic nerve on the injected side annuls this difference. This supports the original view that the toxin is absorbed along nerve routes. These and other experimental results have led Friedemann and his co-workers to conclude that tetanus toxin cannot reach the central nervous system through the capillaries and that it is entirely absorbed along the motor nerves. It is difficult at present to reconcile such conflicting findings and views, and the question arises whether transport of the toxin to the central nervous system may be possible by both routes, neural and circulatory.

In addition to the action on the cord, the toxin affects the brain, which probably explains the general convulsions also characteristic of the disease. This has been shown by injecting toxin into the vitreous humour of rabbits which had previously received an intravenous injection of antitoxin. The animals developed general convulsions with practically no tonic spasm (Permin). According to Sherrington's investigations, disturbance of reciprocal innervation also occurs from the action of the toxin on the brain. Thus, stimulation of an area of the cerebral cortex which in a healthy animal causes opening of the mouth—due to contraction of the depressors of the jaw accompanied by relaxation of the elevators—in a tetanic animal leads to trismus, since the normal inhibition of the closing muscles which form the more powerful group, is now converted into stimulation. An injection of toxin into the posterior nerve routes between the ganglion and the spinal cord does not produce tetanus, but causes severe spasmodic pains (*tetanus dolorosus*).

Tetanus toxin, in addition to acting on the central nervous system, may injure other organs such as the liver, which has been found affected in animals dying in the course of active immunisation against the toxin. Some influence on general metabolism would explain also the intense wasting which occurs in subacute cases of tetanus intoxication in man and animals in spite of free intake of food.

Immunity—Tetanus Antitoxin. It was in the case of tetanus toxin that antitoxic immunity was first demonstrated by Behring and Kitasato, who found that a degree of immunity could be conferred by the injection of very small and progressively increasing doses of tetanus toxin. Subsequent work has shown that the richer a crude toxin is in toxoid, the more useful it is for immunisation procedures. In fact it is doubtful if small animals can be immunised at all by fresh filtrates. In some cases the injection of non-lethal doses instead of commencing an immunity actually increases the susceptibility of the animal. Accordingly, toxin modified by the action of iodine trichloride was originally used. It is found that the greater the degree of

the natural susceptibility of an animal to tetanus, the easier it is to obtain a serum of a high anti-tetanic potency. The horse is, therefore, a most suitable animal and is usually employed. While tetanus antitoxin is frequently present naturally in the blood of cattle, sheep, and goats, it is generally absent from man, horses, pigs, and dogs.

For the immunisation of horses toxin-antitoxin mixtures or formol-toxoid may be injected to begin with and then, after an interval, toxin. The serum of such immune animals possesses the capacity of protecting animals susceptible to the disease against a subsequent injection of a fatal dose of tetanus bacilli or toxin. Further, if injected subsequently to infection, the serum can in certain cases prevent a fatal result, even when symptoms have begun to appear. The degree of success attained depends, however, on the shortness of the time which has elapsed between the injection of the bacilli or of toxin and the injection of the serum. With animals in which symptoms have fully manifested themselves only a small proportion can be saved. As with other antitoxins, there is no evidence that the anti-tetanic serum has any antagonistic effect on the bacilli. It only neutralises the effects of the toxin.

The method now used is similar to that for diphtheria antitoxin (vide Chapter III), mice being generally used. The toxin is complex (see above) and the antitoxin is prepared from the serum of a horse which has been immunised by the injection of a small amount of toxin. The method of preparation is similar to that for diphtheria antitoxin, the toxin being generally used in the form of a suspension in physiological saline.

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The Therapeutic Use of Tetanus Antitoxin. As the result of his experiments, Behring aimed at obtaining a curative effect in the natural disease occurring in man, by the use of antitoxic serum derived from large animals. The essential factors for the success of serum therapy are, first, that there should not be the least unnecessary delay in commencing treatment, and secondly, that the antitoxin should be given in proper amount. This has been demonstrated clearly by experiment. MacConkey determined the amount of antitoxin sufficient to save the life of a guinea-pig when injected under the skin simultaneously, but at another site, with a dose of toxin just sufficient to kill an untreated animal. He then found that one-tenth of this amount of serum sufficed to neutralise 100 fatal doses of toxin when toxin and antitoxin were mixed together *in vitro* prior to injection, and two thousand times as much serum was required to save the life of the animal when injected twenty-four hours after the toxin, i.e. at a time when symptoms of tetanus had already appeared. As has been seen, the toxin rapidly disappears from the blood stream, but even after this has occurred, antitoxin is still up to a point capable of exerting a curative effect. Whether the antitoxin can act by causing dissociation of toxin already fixed to vulnerable cells is, however, doubtful. The serum may be given subcutaneously, intramuscularly,

intravenously, or intrathecally by lumbar puncture. In the first two methods absorption is relatively slow and in the third elimination is relatively rapid. But by the intrathecal method the serum gains rapid access to the grey matter of the cord on which the toxin is exerting its specific action. Sherrington treated monkeys inoculated with tetanus toxin, after the development of symptoms, either by an intrathecal or an intramuscular injection of antitoxin and showed the great superiority of the former method under the conditions studied. It has therefore been recommended that the earlier injections ought to be given both intrathecally and intravenously, the latter route being chosen because antitoxin can be quickly administered, and as Henderson Smith has shown, a high concentration of antitoxin in the body fluids is maintained for a considerable time, the neutralisation of toxin passing out from a focus of infection is thus facilitated. Later, injections should be given by intramuscular and subcutaneous routes, the principle being that,

the form of concentrated antitoxin), repeated doses being given for several days, 20,000 to 30,000 I U being given intravenously along with the first intrathecal dose, and a similar amount injected subcutaneously three or four days later. Yodh has obtained good results from combined intracisternal, intravenous, and intramuscular administration of the antiserum. Though the evidence available is in favour of the combined intravenous and intrathecal administration, some workers have advocated intravenous injection alone in massive doses, e.g. 200,000 units (see Cole). It is pointed out that the irritant action of the serum administered intrathecally may be seriously prejudicial in cases of tetanus (see Florey and Fildes, Paterson, Spaeth).

The results of the therapeutic use of antitoxin in tetanus have not been so good as in the corresponding case of diphtheria. The great difficulty is that an infection is not suspected till the tetanus toxin has already begun to manifest its gravest effects, by which time the nerve cells may have been damaged irreparably. In this connection the experimental results of Roux and Borrel should be recalled, they found that actively or passively immunised rabbits, which were protected against large doses of toxin given subcutaneously, contracted fatal tetanus after a small injection of tetanus toxin injected intracerebrally.

The Prophylactic Use of Tetanus Antitoxin. The practice of giving antitoxin prophylactically in every case of a ragged, unhealthy-looking wound, especially when contaminated with soil, has been generally adopted. The principle had, for a considerable time, been applied in connection with the injuries contracted during the Independence Day celebrations in America, of which tetanus was a not uncommon sequel, a very definite fall in the death-rate was thereby effected. It was during the war of 1914-18, however, that the success of prophylaxis was established. During the early months, in the fighting on the Continent, tetanus was rife—its incidence in the wounded brought to Britain being about sixteen per thousand. After the autumn of 1914 prophylactic injections of antitoxin were given to every wounded man, with the result that the corresponding incidence was reduced to two per thousand. Originally the dose was the equivalent of 1,000 I.U., administered subcutaneously, but now 3,000 I.U. are usually given. As passive immunity is of relatively short duration, the dose should be repeated at seven-days' intervals, if the wound remains unhealed, till three doses have been given. Further, when at later periods operative interference, even with healed wounds, is necessary, a similar dose should be given, either subcutaneously forty-eight

hours, or intramuscularly twelve hours, previous to the operation (care being taken to guard against anaphylactic shock). The effects of the prophylactic use of antitoxin in modifying the clinical type of the disease have been mentioned already. (For combined active and passive immunisation *vide infra*.)

Active Immunisation with Toxoid. This was extensively applied for prophylactic purposes in the recent war. In the British Army two doses each of 1 c.c. of a formol-toxoid preparation were administered at an interval of six weeks. In addition, at the time of wounding tetanus antitoxin was also administered as a further prophylactic measure. In the North African campaign (1940-42) the incidence of tetanus was only 0.13 per 1,000 wounded among the actively immunised, as compared with 1.6 per 1,000 among those who had not been so immunised (Boyd and MacLennan). Active immunisation alone may fail to confer protection either because the individual has responded poorly to the two doses of toxoid or because of an overwhelming infection due to the organisms multiplying in a mass of necrotic tissue. Injections of tetanus toxoid as a rule cause very little disturbance.

Serological Types of Tetanus Bacillus. Cultures of tetanus bacilli which have been washed and heated to deprive them of toxin, when injected into rabbits, lead to the production of agglutinins. By this means it has been shown that the organisms fall into a number of serological types (Tulloch). It is to be noted, however, that there is no difference in the toxins of the different types, as an antitoxin developed by one type neutralises the toxin from other types. Serological differences among strains depend apparently on the H or flagellar antigen.

Bacteriological Diagnosis. The occurrence of 'drumstick' bacilli in stained smears of the discharge from a wound or in necrotic tissue excised from its margin is suggestive, but other organisms with round terminal spores may occur (McIntosh). Again, the tetanus bacillus in the non-sporing condition is not characteristic in appearance. A much more certain method of diagnosis is the production of tetanus by inoculation of material into mice of pathological material directly from the fluid anaerobic cultures. The latter, however, when there are other organisms present beside *B. tetani*. Accordingly, the organisms should be isolated in the pure state by culturing exudates or excised tissues from the wound by Fildes' method. It must be remembered, however, that these procedures take too long a time to afford a useful guide for administration of antitoxin. The history of a contaminated wound should be the indication for treatment.

Further details regarding the bacteriological examination of wounds for sporing anaerobic bacilli, including the tetanus bacillus, are given later under Gas Gangrene.

BACILLUS BOTULINUS (CLOSTRIDIUM BOTULINUM)

One form of 'meat-poisoning' was shown by van Ermengem in 1896 to be caused by the toxin of the anaerobic bacillus to which he gave the name *Bacillus botulinus*. He cultivated the organism from a sample of ham, the ingestion of which in the raw condition had produced a number of cases, some of them fatal. The ham did not show signs of decomposition in the ordinary sense. The symptoms closely corresponded with those in the so-called 'sausage-poisoning', and cases of food-poisoning due to the toxin of *B. botulinus* are now designated 'botulism'. They form a well-defined group, in which the symptoms are referable to the nervous system, and similar

symptoms have been experimentally produced by means of cultures of the bacillus or its toxins. The chief symptoms of botulism are disordered secretion in the mouth and nose, more or less marked ophthalmoplegia externa and interna (dilated pupil, ptosis, etc.), dysphagia, and sometimes aphagia with aphonia, marked constipation and retention of urine, and in fatal cases interference with the cardiac and respiratory centres. Along with these there is practically no fever and no interference with the mental faculties. Vomiting may be an early symptom. The symptoms commence in the human subject usually twelve to twenty-four hours after ingestion of the poison. A considerable number of cases of botulism have now been reported in America, the chief source being home 'canned' meats and vegetables, which have been contaminated by the bacillus and in which it has grown and produced toxin. It can also flourish in meat pickled in brine if the sodium chloride content is under 8 per cent. A few small outbreaks have occurred in Great Britain, the source of the toxin being obscure in certain of those. *B. botulinus* has been cultivated from the intestines of animals, and it has been found to have a wide distribution in America in soils, both arable and virgin. It has also been found in soil from various parts of the Continent and Great Britain.

Several different types of *B. botulinus* occur in nature. Two types designated A and B were first separated by the immunological differences of their toxins (*vide infra*), both cause botulism in man. A third type, C, was later defined, also D and E. C and D are associated with disease in various animals, E has been responsible for human and equine botulism. It has been found that in the eastern states of America most of the outbreaks have been due to type A, whilst in the western states type B has been more frequently found. A similar result has been obtained with regard to the distribution of the two types in the soil in these regions.

MICROSCOPIC CHARACTERS. *B. botulinus* is a bacillus of considerable size, measuring 4 to 6 μ in length and 0.9 to 1.2 μ in thickness, it has somewhat rounded ends and sometimes is seen in a spindle form. It is often arranged in pairs, sometimes in short threads. It is motile and has 4 to 8 lateral flagella of wavy form or they may be more numerous, according to the strain. It stains readily with the ordinary dyes, and is Gram-positive, though not strongly so. Spores are formed, which are oval in shape, terminal or subterminal in position, and only a little thicker than the bacilli.

CULTURAL CHARACTERS AND BIOCHEMICAL REACTIONS. *B. botulinus* can be readily cultivated on the ordinary media, but only under strictly anaerobic conditions. A cooked-meat medium is suitable for cultivation. The earlier workers reported a low optimum temperature (20° to 30° C) for the strains they studied, more recent observations indicate a higher optimum, 35° C. Cultures in *glucose agar* resemble those of certain other anaerobes, there is abundant development of gas, and the medium is split up in various directions. In *glucose gelatin stab culture* a whitish line of growth forms with lateral offshoots, but liquefaction with abundant gas formation soon occurs. In *gelatin plates* the colonies after four to six days are somewhat characteristic; they appear to the naked eye as small semi-transparent spheres, and these on examination under a low power of the microscope have a yellowish-brown colour and are seen to be composed of granules which show a streaming movement, especially at the periphery. On *agar plates*, surface colonies are large and irregular with a thick central nucleus, a thinner periphery, and a fimbriate reticular border. There is considerable variety in the colony appearances, however. The cultures have a rancid, though not foul, odour, due chiefly to the development of butyric acid. *Coagulated serum* or *egg*

white is liquefied by type A and some strains of B, though not by type C. Similarly, in *cooked-meat medium* type A and some strains of B digest the meat and darken it; type C produces no change. Types D and E are likewise non-proteolytic. The *fermentation of sugars* and other carbohydrates varies with the type: A produces acid and gas from glucose, salicin, maltose, and glycerol, and sometimes from lactose but not from saccharose or mannitol; B differs in the absence of salicin fermentation, C does not ferment salicin or glycerol. (For reactions in *milk medium* see Table, p. 451.)

VIABILITY. The spores may in certain circumstances withstand moist heat at 100° C. for one to six hours; in the moist state they withstand 107° C. for fifteen to eighty minutes and 115° C. for four to forty-two minutes (Dickson and others). They are usually killed at 120° C. within five minutes. The results of observations on the heat-resistance of spores have varied somewhat and apparently depend on several factors, e.g. the strain used, age of culture, hydrogen-ion concentration of the medium, etc.; further, only a small proportion of individual spores possesses the maximum resistance to heat, and results depend therefore on the number of spores in the test material.

Toxin and its Pathogenic Effects; Antitoxin. *B. botulinus* has little power of flourishing in the tissues, whereas it produces a very powerful filterable toxin when growing in food-stuffs. When a fairly large dose of toxin is injected subcutaneously in a guinea-pig, symptoms chiefly of paralytic nature may appear within about six hours and death follows within twenty-four hours. *Post mortem* the internal organs show pronounced congestion often with thrombosis and hæmorrhages. When the dose is sub-lethal a somewhat chronic condition may result in which local paralysis forms a striking feature. The characteristic effects can also be produced by oral administration of the filtered toxin, though in this case the dose requires to be larger. Various other animals, e.g. cat, monkey, etc., are also susceptible and exhibit the same effects as those in the human subject. It is noteworthy that this toxin resists the gastric secretion and is absorbed by the stomach and small intestine without undergoing alteration—unlike the tetanus and diphtheria toxins. As in the case of the tetanus poison, the potency of the toxin is remarkable, the fatal dose by subcutaneous injection for a guinea-pig of 250 grms. weight being in some instances 0.0001 c.c. of the culture-filtrate or even less. When massive doses of toxin-free spores are given by the alimentary canal it has been found that multiplication of the organisms and toxin production may follow in certain cases, but there is no evidence that the disease in the human subject is produced in any other way than by the ingestion of previously formed toxin in the contaminated food. The presence of the toxin in contaminated ham was first shown by van Ermengem by inoculation with a watery extract, and a similar result has been repeatedly obtained by others in the case of other articles of diet which have produced the disease. The organism has been recovered from the organs of those who have died of the disease, but it is only after or immediately before death that a few bacilli may enter the tissues.

The condition of the nerve cells in experimental poisoning with the botulinus toxin was investigated independently by Marinesco and by Kempner and Pollack, and these observers agree as to the occurrence of marked degenerative changes, especially in the motor cells in the spinal cord and medulla. On the other hand, Dickson and others have found that the toxin has an important action on the vascular intima, leading to thrombosis in the small vessels, in this way the nerve cells may be damaged or necrosed. The work of Edmunds, Long and Keiper and others indicates, however, that the essential effect of the toxin is on the nerve end-plates of muscles with paralysis of the vagus and parasympathetic nerve endings.

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An antitoxin was prepared by Kempner by the usual methods, and was shown not only to have a neutralising property, but to have considerable therapeutic value in experimental animals when administered some hours after the toxin. These results have been fully confirmed. Leuchs showed that the combination toxin-antitoxin can be split up by the action of acids and the two components recovered, just as Morgenroth showed to occur with diphtheria toxin. The various types of *B. botulinus* (*vide supra*) differ in the antigenic characters of the toxins which they produce. Thus the antitoxin to toxin A has no effect on toxin B, and *vice versa*. This is, of course, an important matter in relation to treatment, and a mixture of antitoxins for the A and B types has been used for therapy in human cases. The practical value of antitoxin for therapeutic purposes is limited owing to the fact that by the time the condition is clinically recognised irreparable toxic effects have already been produced.

Botulism in Animals. The disease occurs in natural conditions in fowls and is known as 'limber-neck', in this affection types A and B have been found. Fowls are very susceptible to the toxin of type A, dying within twenty-four hours after oral administration. Type C has been responsible also for paralytic disease of chickens (Bengtson), the ingestion of the larvae of carrion flies harbouring the organism is apparently the cause of the condition. Type C likewise causes botulism in ducks under natural conditions. This type has also been reported in forage poisoning of cattle in Australia (Seddon), but a distinction has been drawn between the strains of type C associated with this condition and those responsible for disease in chickens and ducks, the latter being designated type C_a and the former C_b . The difference consists in the ability of the C_a antitoxin to neutralise C_a and C_b toxins whereas the antitoxin for C_b does not neutralise the C_a toxin. Type D has been found to cause 'lamsiekte', a disease of cattle in South Africa associated with phosphorus deficiency and a resulting pica which leads to these animals eating the bones of carcasses on the veldt in the muscle tissue attached to the

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The investigation of cases and outbreaks is aided with the examination of suspected articles of food. An extract in saline solution is made from the material and injected into mice or guinea-pigs. Control animals are also

Wound infection by the anaerobes received further study in the recent war, though gas gangrene was a less frequent and serious complication of wounds than in the war of 1914-18. MacLennan's results in the North African campaign are illustrative. He classified such wound infections into (1) simple contamination, (2) anaerobic cellulitis, and (3) anaerobic myositis (true gas gangrene). Simple contamination was present in 20 to 30 per cent of all wounds. Most of the species were of low pathogenicity and did not persist, these were mainly *B. butyricus*, *B. tertius*, and *B. sporogenes*, but *B. welchii* was also fairly prevalent. Anaerobic cellulitis occurred in about 5 per cent of contaminated wounds, especially if there was extensive laceration of the soft tissues other than muscle, the bacilli then multiplying in the necrotic tissue. The condition was either localised or extensive and the onset was gradual as compared with typical gas gangrene, while the systemic effects were usually slight. The most frequent species were *B. sporogenes*, *B. welchii*, and *B. tertius*. Typical gas gangrene occurred in about 0.4 per cent of all wounds. It was of sudden onset and the infection spread with great rapidity, especially in muscle, and was associated with the characteristic symptoms. The species of organisms present were in order of prevalence *B. welchii*, *B. oedematiens*, *Vibrio septique*, *B. histolyticus*, *B. bifermens*, *B. fallax*, and *B. tetani*. Mixed infections by several of these organisms were not infrequent. The mortality was high. In the desert campaign the area over which the fighting took place was, of course, uncultivated ground and in most cases the source of infection was probably contaminated clothing. When the fighting moved to the agricultural land of Tripolitania and Tunisia the incidence of gas gangrene was about twice that of the desert campaign, but anaerobic cellulitis was less frequent, probably due to the greater efficiency of wound treatment. The mortality was also relatively low (30 per cent.) as a result of accurate diagnosis, improved surgical measures, and intensive serum treatment.

BACILLUS WELCHII (CLOSTRIDIUM PERFRINGENS)

This organism was first described by Welch and Nuttall in 1892, who showed that it was the cause of the extensive gaseous development which sometimes occurs in the organs *post mortem*, resulting in the formation of rounded gas cavities. It is now recognised that it is identical with an organism cultivated later by E. Fraenkel and called by him *B. phlegmonis emphysematosæ*. The same bacillus was described by Veillon and Zuber, who gave it the name *B. perfringens*. During the war of 1911-18 it came into great prominence, as it was proved to be by far the most important agent in the production of gas gangrene.

MICROSCOPIC CHARACTERS. As seen in the serous fluid in a case of spreading gas gangrene, it is a comparatively large bacillus, measuring usually 4 to 6 μ in length (Figs. 111, 112) and relatively broad, about 1 μ , but the thickness varies somewhat. Its ends tend to be rounded, although some of the shorter forms are almost rectangular. In cultures (Fig. 114) it is rather pleomorphic, and in sugar-free media there is a tendency to form filaments (Henry), short, almost coccus-like forms, may also be met with. It is readily stained with the basic dyes, and is Gram-positive, though in older cultures Gram-negative forms occur. In the tissue fluids it usually has a distinct and fairly broad capsule (Figs. 113, 115), sometimes, however, no capsule is seen. In agar or broth media, no capsule is seen ordinarily, but in serum media it is conspicuous. The organism is non-motile, and no flagella have been demonstrated.



FIG. 111 Film taken from margin of spreading gas gangrene, showing numerous examples of *B. welchii* (pure)¹ Gram's stain $\times 1,000$



FIG. 112 Film from necrosed muscle in gas gangrene, showing a few *B. welchii* with remains of muscle fibres Gram's stain $\times 1,000$.

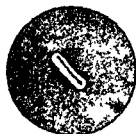


FIG. 113 *B. welchii* showing capsule. Losm capsule method $\times 1,000$



FIG. 114 Film from a pure culture of *B. welchii* Gram's stain $\times 1,000$

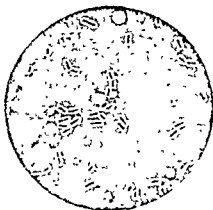


FIG. 115 *B. welchii*, showing capsules, film preparation from bone marrow in a case where gas cavities were present in the organs. $\times 1,000$

¹ We are indebted to Prof. J. W. McNee for the preparations from which figs. 111 and 112 were made.

In the spreading area of the disease no spores are found though they have been described in the later stages when the bacillus is associated with other organisms. At first it was believed not to form spores, but spores are produced in serum media (Dunham); they are oval and fairly large, usually subterminal, occasionally central. In ordinary media, however, and in the presence of sugar, no spores are formed. The acid produced by fermentation of sugar inhibits spore formation.

CULTURAL CHARACTERS AND BIOCHEMICAL REACTIONS. *B. welchii* grows readily on various media, but only under fairly strict anaerobic conditions. Growth is enhanced by the presence of fermentable carbohydrate and by blood or serum. It flourishes best at the temperature of the body, but grows also at room temperature. On agar the *superficial colonies* are usually circular in form, moist in appearance, opaque, with smooth surfaces and regular margins, there being no radiate outgrowth or downgrowth (Fig. 116); the *deep colonies* are usually oval or lenticular in form with sharp outline. Surface colonies sometimes have a transparent border with radial striations. Colonies on blood agar show well-marked zones of hæmolysis. *B. welchii*

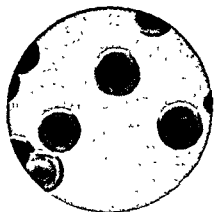


FIG. 116 *B. welchii* surface colonies from anaerobic culture on glucose agar after forty-eight hours at 37° C. $\times 5$

liquefies gelatin but not coagulated serum. Certain strains, however, derived from animal diseases, have been stated to liquefy serum. In milk the characters of the growth are of importance; the organism grows rapidly and leads to coagulation of the medium, the clot becomes broken up by gas bubbles—the so-called 'stormy clot reaction'—and ultimately forms irregular tough masses bathed in comparatively clear whey. While this is a fairly constant character, some strains may fail to give it. (Stormy fermentation of milk is also produced by certain anaerobic butyric bacteria which form terminal oval spores—McCoy *et al.*) There is no digestion of the casein even after a long time. The culture has an

odour of butyric acid. In *cooked-meat medium*, *B. welchii* renders the meat a pink colour and produces a considerable amount of gas; there is a sour smell, but no putrid odour or blackening of the medium. It produces acid and gas from glucose, lactose, saccharose, maltose, and starch, and sometimes from glycerol and inulin. Fermentation of these substances takes place with great rapidity, but the acid formed has a markedly deterrent action on the growth, and soon leads to its cessation. Dulcitol and mannitol are not fermented. Usually salicin is not acted on, but sometimes fermentation occurs. *B. welchii* has thus very active saccharolytic action, whereas its digestive effect on proteins is slight, and these properties will be found to have an important bearing on its pathogenic effects.

VIABILITY. Cultures in media containing fermentable sugars remain viable for only a few days; the acid produced is bactericidal and spore production is arrested. In sugar-free media cultures remain viable for several months. In the presence of moisture the spores are generally killed at 100° C. within five minutes.

Pathogenic Effects. In addition to invading the blood stream about the time of death, and giving rise to gas cavities in the organs *post mortem*, *B. welchii* has been found in various emphysematous and gangrenous conditions

in which the infection starts in connection with the alimentary canal, e.g. certain forms of appendicitis and cholecystitis. It may also be associated with puerperal infection and particularly septic abortion. As has been indicated, it is the most frequent cause of gas gangrene from war wounds. In this condition it is now recognised that the starting-point is usually lacerated muscle, which has become contaminated with soil or clothing containing the organism. The spread of the disease is often remarkable, as cases have been recorded in which extensive emphysematous swelling with gangrene of a limb has occurred with a fatal result well within twenty-four hours. In some cases the infection may be confined to individual muscles. Within a muscle, the necrotic change may affect individual fibres, leaving others in relation to them unaffected. The stages as described by McNee and Shaw Dunn are as follows. The bacilli spread with great rapidity along the interstitial tissue of the muscle, and may be found beyond the actual site of gangrene. They are present often in very large numbers and in practically pure culture. The fibres thus surrounded become somewhat swollen, altered in staining reaction, and separated from the interstitial tissue by a zone of serous fluid, poor in protein. The fibres then become completely necrosed, the sarcolemma nuclei losing their staining reaction, and about this time the fluid within the sarcolemma contains the bacilli in large numbers. There is then evolution of gas, chiefly from the muscle carbohydrates, and the muscle substance becomes broken up and disintegrated, though the transverse striation may persist for a considerable time (Fig 112). Finally the dead muscle may be invaded by other organisms, and become putrid and softened. Along with these changes in the muscle there occur œdema and emphysema in the interstitial and subcutaneous connective tissue, while the skin shows various kinds of discoloration, and the affected part is swollen, tense, and gives crackling on palpation. To the naked eye the affected muscle is at first swollen and pale and has lost its elasticity, it soon assumes a brownish-red colour, while later it is rotten, how-
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flourish in the necrotic tissue.

The spread of the bacilli is attended by practically no leucocyte reaction, unless when the invasion is becoming arrested or when it takes place around other organisms. The growth of the bacilli is essentially local, but they may enter the blood shortly before death, when they have been found in a certain proportion of cases. Instances have also been recorded in which they have settled in other parts of the body and produced lesions there—the so-called metastatic gas gangrene.

Apart from typical gas gangrene, *B. welchii* may be associated with the condition described by MacLennan as 'anaerobic' cellulitis (*vide supra*).

Most of the local and systemic effects of *B. welchii* are due essentially to the exotoxins produced by this organism, these are dealt with later, and also the different types of the organism in relation to diseases of sheep.

It has been observed that the number of *B. welchii* in the bowel may undergo great increase in certain conditions, e.g. the achlorhydria of pernicious anaemia (Davidson), intestinal obstruction, and it has been suggested that the toxin of the organism may be absorbed from the intestine and be responsible for pathological effects in the latter condition. It is doubtful, however, whether *B. welchii* toxin is absorbed in an active state from the intestine.

Experimental Inoculation. The virulence of strains of *B. welchii* varies considerably. In the case of many strains fatal gas gangrene may be produced in guinea-pigs by injection of cultures in fluid media, the bacterial invasion being favoured by the toxin present. The bacilli by themselves, for example, when obtained from a surface growth on a solid medium, do not produce infection, but Bullock and Cramer showed that when an ionisable calcium salt is injected, the bacilli quickly invade the tissues affected by the salt and cause a rapidly fatal infection (see Evans). In the experimental disease the bacilli are abundant locally, but only a few are present in the blood stream. When the dose is sublethal a local gangrene may occur, with subsequent separation of the dead tissue; thereafter healing may rapidly follow. Intramuscular injection is the most effective method, especially in the rabbit, which is more resistant than the guinea-pig. The pigeon is found to be the most susceptible of the animals hitherto tested, the lethal dose being only a fraction of that for the guinea-pig. Injection into the pectoral muscle of a pigeon causes lesions in the muscle closely resembling those in gas gangrene in man, and death follows very rapidly, sometimes within a few hours.

Toxins and Biological Types of *B. welchii*. It has been long recognised that this organism is an active producer of diffusible toxins. Bull and Pritchett showed that a culture-filtrate injected into various animals led to local lesions closely resembling those caused by the bacilli themselves, e.g. œdema and necrosis of muscle; and the general systemic effects of gas gangrene are undoubtedly due to a true toxæmia. Culture-filtrates are also markedly hæmolytic and *in vivo* the hæmolysin may produce hæmoglobinuria. Injected intravenously in animals these filtrates are lethal, and *post mortem* focal necrosis of the liver and kidney is observed. Culture-filtrates also contain leucocidin, fibrinolysin (Reed *et al.*), and a spreading factor (Behring, McClean) similar to that of streptococci, viz hyaluronidase.

The study of strains of the organism isolated from animal diseases and the toxins produced by them has brought to light the existence of biological types of *B. welchii* differing in their production of particular toxic fractions. The classical type found in the intestine of man and animals and in gas gangrene is now designated type A. Another variety, type B, is the causal organism of lamb dysentery (Dalhng), type C, sometimes called *B. paludis*, is responsible for 'struck' of sheep, a toxæmic condition due to the absorption of toxin from the small intestine (McEwen); a fourth variety, type D (*B. ovitoxicus*) is associated with a condition of infectious entero-toxæmia of sheep, including the so-called 'pulpy kidney' disease of lambs. These four types are differentiated according to the toxic components present in culture-filtrates (Wilsdon). Seven such toxins, designated α , β , γ , δ , ϵ , η , θ , have been defined (Glenny *et al.*, Ipsen *et al.*; Prigge). The production of these by the four biological types and their toxic effects are summarised as follows:

- Type A— α , η , θ (α predominant)
- .. B— α , β , γ , δ , ϵ (β and ϵ predominant).
- .. C— α , β , γ , δ (β and δ predominant).
- .. D— α , ϵ (ϵ predominant).

It should be noted, however, that the production of these fractions is not completely stable; thus strains of D may lose the property of producing toxin ϵ (Borthwick).

The toxic components have the following effects :

Toxin α —hæmolytic, lethal, necrotising, and acts as a lecithinase.

„ β —lethal, necrotising.

„ γ —lethal

„ δ —hæmolytic, lethal

„ ϵ —lethal, necrotising

„ η —lethal

„ θ —hæmolytic, lethal, sometimes described as necrotising, but producing rather a hæmorrhagic condition, does not act as a lecithinase

The hæmolytic property can be demonstrated with sheep's erythrocytes, but in the case of the θ component, with horse's red cells (Evans) ; the necrotising effect by intradermal injection in guinea-pigs ; and the lethal action by intravenous injection of mice. The lecithinase effect is manifest in the so-called Nagler's reaction, i.e. a characteristic opalescence or turbidity is produced in human serum when the toxin is added and this effect is specifically neutralised by antitoxin, the same reaction occurs with an extract of egg-yolk and is due to the splitting of lecithin into phosphocholine and a glyceride. It is of interest that there is an antigenic relationship between the θ toxin and the O lysin of streptococci (Todd). The α toxin is relatively thermostable while the β and θ fractions are thermolabile.

It has also been shown that muscle tissue when incubated with *B. welchii* type A undergoes disintegration due apparently to a collagenase which attacks the reticulin scaffolding. The pulping of muscle observed in gas gangrene may be due to this product, and Oakley and his associates have classified it as an additional toxic component, designated κ , immunologically distinct from the α and θ toxins and hyaluronidase.

All these toxins are specifically neutralised by appropriate antitoxic sera and the different biological types may be identified by protection tests in mice injected intravenously with culture fluid and antitoxic sera. Thus :

A antitoxin neutralises only type A toxin

B „ „ types A, B, C, and D toxins

C „ „ types A and C toxins

D „ „ types A and D toxins

Antitoxin and Immunity. By injecting carefully graduated doses of the toxin into animals, e.g. horses, an active immunity can be produced, and the serum of the treated animals possesses antitoxic properties. The antitoxin neutralises all the effects of the toxin in multiple proportions, and is protective and curative against infection with the bacillus in experimental animals. The antitoxin is titrated by its capacity to neutralise the toxin when tested by intravenous injection in mice (Hartley) or by intracutaneous injection in rabbits or guinea-pigs. An international unit has been accepted for standardisation, viz. the equivalent of 0.322 mgm. of a dried standard antitoxin prepared in the National Institute of Health, Washington, U.S.A. In the recent war, the dose recommended for prophylactic purposes was 9,000 units given intravenously where practicable or alternatively by intramuscular injection, and the therapeutic dose was three times the prophylactic dose given intravenously and repeated at intervals of four to six hours. (Usually a polyvalent antitoxin is administered containing also antitoxin for *V. septicæ* and *B. oedematiens*—*vide* pp. 442, 444.) Antitoxin was extensively used in the war for prophylactic purposes and regularly applied in the

treatment of cases of gas gangrene. Though the value of the antitoxin has not been fully established by statistical evidence, the data available all indicate its efficacy, provided the cases receive at the same time satisfactory surgical treatment. The antitoxin has also been used for the treatment of gangrenous appendicitis and puerperal infections; it has been given prophylactically before operations on the alimentary or the genito-urinary tracts.

Antitoxic sera have also been applied in the control of the animal diseases referred to above. Thus effective protection against lamb dysentery can be conferred by passive immunisation with antitoxin for type B; alternatively, active immunisation of the pregnant ewe may be adopted, toxoid being used; the resultant antitoxin is conveyed to the lamb in the colostrum. Immunisation with the appropriate toxoid is of value in protecting against 'struck', while type D toxoid can be used in the pregnant ewe for protection of the lamb against 'pulpy kidney' disease.

The antigenic structure of the bacilli has been studied by agglutination reactions and these reveal considerable heterogeneity even within the biological types distinguished by their toxic components. The specificity of the agglutination reactions depends both on the capsular substances which are probably of polysaccharide nature and on specific O antigens. S→R variation occurs and this is associated with loss of specific O antigen (see Henderson).

VIBRION SEPTIQUE (*CLOSTRIDIUM SEPTICUM*)

The *Vibrion septicus* was first discovered in putrefying carcases and so named by Pasteur. He described its characters and the lesions produced by it. He found that it grew only in anaerobic conditions, but pure cultures were not obtained. A similar organism was later studied by Koch, and named the 'bacillus of malignant oedema'. It is not possible to say whether

the organisms originally described by Pasteur and by Koch were identical. *V. septicus* is an inhabitant of the intestine in animals and is found in soil.

This organism is not so frequently present in gas gangrene as *B. welchii*, though in the war of 1914-18 it attained a fairly high prevalence. Weinberg pointed out that cases of pure infection were rare, and that in these emphysema was not a striking feature, gas occurring only in the deeper tissues in small bubbles, and sometimes only recognisable at operation. In a fatal case observed by Mur in which the bacillus was present in pure condition there occurred intense oedema with swelling and induration of the tissues, and the formation of vesicles on the skin. These

FIG. 117. Film preparation from the affected tissues in a case of malignant oedema due to *V. septicus* showing the spore-bearing bacilli. Gentian-violet. $\times 1,000$.

changes were attended with a reddish discoloration afterwards becoming livid. Emphysema was not recognisable until the very tense limb was incised, when it was detected, though in small degree. Further, the tissues had a peculiar heavy, but not putrid odour. The bacillus was present in enormous numbers in the affected tissues. Reference has already been made to the occurrence of the organism in gas gangrene in the recent war.

MICROSCOPIC CHARACTERS. *Vibrion septicus* usually occurs in the form of single rods 3 to 10 μ in length and 0.6 to 1 μ broad, but both in the

tissues and in fluid cultures it frequently grows into long filaments, which may be uniform throughout or segmented at irregular intervals. In cultures on solid media it occurs mainly in the form of shorter rods with somewhat rounded ends. It can be readily stained by any of the basic aniline stains and is Gram-positive, but in older cultures Gram-negative forms occur. The bacilli are motile, possessing several peritrichous flagella. Motility is usually well marked in the serous exudate of the lesions, but in cultures only a few individuals show active movement. Under suitable conditions spores are formed which have an oval shape, their thickness exceeding that of the bacillus, they are central or subterminal in position. Spore formation occurs in cultures above 20°C ., and is usually well seen within forty-eight hours at 37°C . In acute spreading lesions the bacilli are usually free from spores, but at a later period they may be found, and large swollen lemon-shaped forms are often seen, the so-called 'citron bodies'.



FIG. 118 *Vibrio septique*, surface colonies from an anaerobic culture on glucose agar after forty-eight hours at 37°C $\times 5$



FIG. 119 Stab cultures in agar, five days' growth at 37°C . Natural size

A *Tetanus bacillus* B *Vibrio septique* C *Bacillus chaetius*

CULTURAL CHARACTERS AND BIOCHEMICAL REACTIONS. This organism is a strict anaerobe, it multiplies at room temperature, but the optimum is 37°C . It grows on ordinary media, but better in the presence of a fermentable carbohydrate, e.g. glucose. In stab culture in glucose agar, growth is rapid. Along the line of puncture, growth appears as a somewhat broad whitish line, with short lateral projections here and there (Fig. 119, B). Gas may be formed, but this is most marked in a shake-culture. Surface colonies on agar are large, irregular, and somewhat transparent with fimbriate borders due to filamentous projections, not unlike those of the tetanus bacillus (q.v.), though of somewhat coarser character (Fig. 118). Deep colonies when fully grown, exhibit also filamentous projections, giving them a woolly appearance. Colonies on blood agar show haemolytic zones. In gelatin liquefaction occurs. Coagulated serum is not liquefied. In cooked-meat medium the meat is

reddened, but there is no digestion. The cultures have a peculiar heavy, though not putrid, odour. Glucose, lactose, salicin, and maltose are fermented, but not saccharose, mannitol, inulin, glycerol or starch. In milk acid and some gas are produced and there is slow

clotting. The organism is thus mainly saccharolytic and only weakly proteolytic.

Experimental Inoculation. A considerable number of animals—the guinea-pig, rabbit, mouse, dog, sheep, goat, and pigeon, for example—are susceptible to inoculation with this organism. There is general agreement as to its marked pathogenic properties. Especially is this the case when inflammatory exudate containing the bacillus is used for inoculation, a mere fraction of a cubic centimetre being a fatal dose. McIntosh found that 0.01 c.c. of a fluid culture injected intramuscularly killed a guinea-pig within twenty-four hours.

Subcutaneous inoculation with pure cultures produces in the guinea-pig chiefly a widespread gelatinous oedema, and a blood-stained serous fluid exudes from the affected part. The underlying muscles are softened and partly necrosed, and of bright red colour; but there is little formation of gas, and putrid odour is almost absent. The internal organs show little change. The bacilli are present in the peritoneal fluid and on the peritoneal surface of the liver, and occur as long motile filaments and 'citron bodies' (*vide supra*). They have sometimes been cultivated from the blood, but they are always scanty.

When the bacilli are injected into mice, however, they enter and multiply in the blood stream, and are found in considerable numbers in the various organs (see Henderson and Gorer), while the spleen is much enlarged.

Toxin. Exotoxins can be obtained in fluid cultures under suitable conditions. Of a potent culture-filtrate 0.1 c.c. injected intravenously into a rabbit will cause death in three to fifteen minutes, with respiratory disturbance, paralysis, and convulsions. Guinea-pigs and mice are also killed by the filtrate given intravenously. Intramuscular or subcutaneous injections cause local oedema, red staining of the tissues, and necrosis. Intracutaneous injection in guinea-pigs causes a necrotic lesion. *In vitro* the toxin causes contraction of smooth muscle. Culture-filtrates are hæmolytic and also contain hyaluronidase.

Occurrence in Animal Diseases. Braxy is a disease of sheep due to infection by *Vibrio septique*. In this condition there is an intense inflammatory lesion with oedema, hæmorrhage, and necrosis, starting in the wall of the fourth stomach and tending to spread to neighbouring parts of the alimentary tract. The bacilli can be demonstrated in the affected tissues. Little is known, however, regarding the factors that predispose to this infection. Culture-filtrates, toxin-antitoxin preparations, and formalised whole cultures ('anaculture') have been utilised with success in the prophylactic immunisation of sheep. This organism may also be responsible for 'blackleg' in sheep and cattle.

Immunity. Roux and Chamberland (1887) showed that if guinea-pigs were injected repeatedly with non-fatal doses of cultures sterilised by heat or freed from the bacilli by filtration, immunity against the living organism could be developed in a comparatively short time. They found that the filtered serous exudate of animals dead of the disease also gave immunity when injected in small doses. This is one of the first diseases to which immunity was produced by injections of toxins. The serum of immunised animals contains antitoxin, which can be titrated according to its capacity to neutralise the action of the toxin as tested by intravenous injection in mice or intracutaneous injection in guinea-pigs (Hartley and Bruce White). The international unit of the antitoxin is of equivalent potency to that of 0.2377 mgm. of a dried standard antitoxin kept at the National Institute for Medical Research, London. The antitoxin serum protects effectively against

inoculation with the living bacilli or washed spores plus calcium chloride, it also has curative action (Craddock and Parish). The antitoxin has been used therapeutically and prophylactically in the human subject, usually incorporated in a polyvalent preparation containing also antitoxins for *B. welchii* and *B. oedematis*. The prophylactic dose recommended of *V. septique* antitoxin is 4,500 international units given intravenously if possible and the therapeutic dose three times the former, given intravenously and repeated at intervals of four to six hours.

V. septique may be identified by the specific neutralisation of its hæmolysin for human red blood corpuscles with antiserum (Hayward and Gray).

Antigenic structure. Henderson (1934) has recognised four groups of the organism differentiated by their O antigens and these can be divided further by differences in their H antigens. Some serological relationship with *B. chauvæi* has been demonstrated.

BACILLUS CHAUVÆI (CLOSTRIDIUM CHAUVÆI)

This bacillus, so far as is known, never infects the human subject. It causes quarter-evil (German, Rauschbrand, French, Charbon Symptomatique). The natural disease, which occurs especially in certain localities, affects chiefly young cattle, also sheep. Infection takes place by some wound of the surface, and then spreads in the region around, attended by inflammatory swelling, bloody œdema, and emphysema of the tissues. The part becomes greatly swollen, and of a dark, almost black, colour. Hence the name 'black-quarter' by which the disease is often known. The bacillus is present in large numbers in the affected tissues, associated with other organisms, and also occurs in small numbers in the blood of internal organs. Morphologically it closely resembles *V. septique*. It does not usually appear, however, in long filaments when films from the surface of the liver of an infected guinea-pig are examined, occasionally it occurs in short chains. The characters of the cultures also resemble those of *V. septique*. Its fermentation reactions are similar to those of *V. septique*, except that, unlike the latter, it ferments saccharose, but not salicin. Stress was at one time laid upon certain differences in cultural characters for distinguishing it from this organism. Weinberg concluded, however, that it bears to *V. septique* a similar relation to that of types B, C, and D of *B. welchii* to type A. Agglutination tests show heterogeneity of strains in their antigenic structure and only slight relationship with *V. septique*, though complement-fixation reactions indicate a close relationship.

The disease can be readily produced in various animals, e.g. guinea-pigs, by inoculation with the affected tissues of diseased animals, and also by means of pure cultures, though an intramuscular injection of a considerable amount of the latter is sometimes necessary. The condition produced in this way closely resembles that in gas gangrene. Exotoxin is formed in culture, it is lethal to mice on intravenous injection and on subcutaneous injection of guinea-pigs produces a local œdema. The disease is one against which immunity can be developed in various ways, and methods of preventive inoculation have been adopted in the case of animals liable to suffer from it e.g. by injection intravenously or intraperitoneally of a non-fatal dose of the œdematous fluid ('aggressin') from the tissues of infected animals containing the bacilli, or by injection of larger quantities of this material attenuated by heat, drying, etc. (Arlong, Cornevin and Thomas, and others). Cultures attenuated by heating or products of the bacilli obtained by filtration of cultures have also been used. Formalised broth cultures have likewise

properties, sometimes forming acid, but no gas, from glucose and maltose alone among the usual fermentable substances. Its action on milk and coagulated serum is similar to that of the *B. sporogenes* (*vide infra*), but is even more rapid. In meat medium also it produces very rapid digestion, and one feature described by Henry is the separation of white balls of acicular crystals which are probably tyrosine—an appearance which is apparently characteristic of this organism. Striking evidence of the proteolytic action of this organism is seen when it is injected subcutaneously in a guinea-pig. A rapid digestion of the tissues in the vicinity occurs, so as sometimes actually to expose the bones. A soluble toxin has been demonstrated in young cultures

BACILLUS TERTIUS (CLOSTRIDIUM TERTIUM)

in contaminated wounds, and be third in order of frequency among and thin bacillus, and is often somewhat curved, it is Gram-positive, but the power of retaining the stain is soon lost in cultures. It is feebly motile. The spores are terminal, the small forms are round, and stain deeply with a basic dye, the larger are oval, racquet-shaped, sometimes of considerable length. The organism is micro-aerophilic rather than strictly anaerobic. The superficial colonies are round, transparent disks, which do not become large; the deep colonies are of lenticular shape, occasionally, from both, small offshoots occur. On a moist surface there is a tendency for the growth to spread as a thin film. In milk a small amount of gas is produced, and a day or two later a soft friable coagulum. In cooked-meat medium acid and gas are formed and the meat assumes a pink colour. There is no liquefaction of gelatin or coagulated serum. The organism has wide fermentative action when tested on various carbohydrates, fermenting glucose, lactose, saccharose, salicin, mannitol, and xylose, but different strains may vary. It has practically no pathogenic effects when tested experimentally, though it probably gives rise to gas formation in wounds.

BACILLUS BUTYRICUS (CLOSTRIDIUM BUTYRICUM)

This organism occurs in the form of bacilli which are about 3 to 4 μ long by 0.7 μ broad, but showing some variation in length. It is motile and has peritrichous flagella. The spores are oval and subterminal. Capsules are observed in culture. The bacilli store glycogen and stain yellow with iodine; they are Gram-positive. The organism is a strict anaerobe and grows best on media containing fermentable sugar. The optimum temperature is 30° to 40° C. Surface colonies are small, greyish-white, semi-opaque, with a circular outline. Neither gelatin nor coagulated serum is liquefied. In milk clotting occurs. In meat medium there is no digestion. Glucose, lactose, maltose, saccharose, salicin are fermented, mannitol and glycerol are fermented by some strains. The organism occurs in soil and has been isolated from infected wounds, but is non-pathogenic and does not produce toxin.

BACILLUS SPOROGENES (CLOSTRIDIUM SPOROGENES)

This organism, which was first separated from faeces by Metchnikoff and described by him, is probably the commonest anaerobe in cultivated soil. It is present in the great majority of putrid wounds, and, owing to its rapid growth and spore formation, often interferes with the separation of other

anaerobes. It is a fairly large bacillus, of about the same length as *B. welchii*, but thinner, and usually occurs as single elements. It is Gram-positive, but, as is common with members of the group, Gram-negative forms are to be found in older cultures. Spore-bearing forms are common in wounds, and in culture spores are formed with great rapidity, so that they may be seen within twenty-four hours (Fig. 120). The spores, which have very high powers of resistance, are oval and usually subterminal, though occasionally central in position. The organism possesses numerous peritrichous flagella, and most strains are actively motile. It grows readily under anaerobic conditions but is not a strict anaerobe. Cultures have a markedly putrid odour. In stab culture in glucose agar the growth forms a thick line, from which there are short and stout lateral offshoots, attended by abundant gas formation, while individual colonies are small balls with woolly margin. Superficial colonies have a granular centre and present an arborescent or feathery appearance at the edge. (When the medium is dry the colonies are of the 'medusa-head' type like those of the anthrax bacillus.) The organism rapidly liquefies gelatin and coagulated serum. In cooked-meat medium there is evolution of gas and rapid digestion, the meat assumes a dirty, purplish tint, and ultimately becomes blackened. In milk there is a precipitation of casein without actual coagulation, and then digestion rapidly follows. The organism ferments glucose, levulose, and maltose, but usually none of the other sugars ordinarily employed. The organism is thus seen to have marked proteolytic properties, and it has been shown to form amino-acids, and as final products ammonia, sulphuretted hydrogen, and various volatile substances. It forms large quantities of butyric acid even in sugar-free media (Wolf and Harris).

B. sporogenes has little or no pathogenic properties when injected in animals, and observations on war wounds supply no evidence that it invades the healthy tissues. It may be regarded as a proteolytic saprophyte which grows on dead and dying tissues and brings about digestive softening and putrefactive changes.

The following other organisms of the group of sporing anaerobic bacilli have also been described, mostly in infected wounds or gas gangrene.

B. parasporgenes (*Cl. parasporgenis*) differs from *B. sporogenes* in producing a smooth round surface-colony, and can also be distinguished by agglutination reactions with antisera (McIntosh).

B. bifementans (*Cl. bifementans*) is mainly proteolytic. It is motile and has peritrichous flagella. Spores are formed readily and are oval and subterminal. Surface colonies are round or crenated, deep colonies are without outgrowths. In other characters it resembles *B. sporogenes*. Some strains are toxicogenic, these are designated *B. sordellii*.

B. aerofetidus (*Cl. aerofetidum*) is also essentially proteolytic. It appears as a slender bacillus 2 to 1 μ in length, which is only slightly motile and tends to stain Gram-negatively. Spores are not readily produced, when seen they are subterminal in position. Surface colonies are small (1 to 2 mm. in

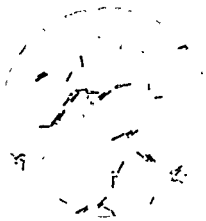


FIG. 120. *B. sporogenes*, pure culture, showing subterminal spores. Stained with carbol-thionin $\times 1,000$.

diameter), round, and transparent. It is non-pathogenic to laboratory animals.

B. multifерmentans tenalbus (*Cl. multifерmentans*) possesses saccharolytic but no proteolytic properties (like *B. fallax*). In morphology it resembles *V. septique* but differs in its lack of pathogenic action when injected into animals. Surface colonies are large and have irregular margins; when fully grown they are raised and opaque.

B. cochlearius (*Cl. cochlearium*) is a motile bacillus showing oval terminal spores, and differs from other members of the group in possessing neither saccharolytic nor proteolytic properties.

B. tetanomorphus (*Cl. tetanomorphum*) is of interest in its close morphological similarity to *B. tetani*. Surface colonies are small, more or less round, and transparent. It is non-pathogenic to laboratory animals. It possesses saccharolytic but no proteolytic properties.

B. sphenoides (*Cl. sphenoides*) also tends to resemble *B. tetani* in morphology when the spores are fully developed. The bacilli are motile and may appear fusiform in shape. Surface colonies are round and without projections. It possesses saccharolytic but no proteolytic properties.

B. capitovalis (*Cl. capitovale*). This organism has been found *post mortem* in cases of septic infection in the human subject and it has also been isolated from faeces. It forms spores which are oval and terminal. The surface colonies are very small and transparent with more or less circular borders. It is saccharolytic and slightly proteolytic. Glucose is fermented, but not lactose, saccharose, salicin, or maltose. It liquefies gelatin, but not coagulated serum. In meat medium it produces blackening and a putrefactive odour. It is non-pathogenic.

B. hastiformis (*Cl. hastiforme*). Surface colonies are very small, transparent, and at first circular, later irregular. It liquefies gelatin but not coagulated serum. It is non-saccharolytic (see MacLennan, 1939).

B. putrificus (*Cl. putrificum*) was originally isolated from faeces. It has the general characters of the group, but tends to form long threads or filaments in older cultures. Spores are not usually seen; when present they are round and terminal. Surface colonies are transparent with fimbriate margins. It is actively proteolytic and non-saccharolytic. In meat medium blackening occurs and the culture has a putrefactive odour. Gelatin is liquefied and there may be liquefaction of coagulated serum. It is non-pathogenic (see MacLennan, 1939).

B. paraputrificus (*Cl. paraputrificum*) has been found in faeces. It has terminal oval spores. It is saccharolytic but not proteolytic. Glucose, lactose, saccharose, salicin, and maltose are fermented but not mannitol. It is non-pathogenic.

B. difficilis (*Cl. difficile*) has been isolated from faeces. Surface colonies are irregular and rough. Some strains liquefy gelatin, but there is no liquefaction of coagulated serum. Glucose, salicin, and mannitol are fermented. Some strains produce exotoxin which is lethal to various laboratory animals.

B. carnis (*Cl. carnis*) has been isolated from soil. It is not a strict anaerobe. Surface colonies are transparent, circular, and become lobate. It does not liquefy gelatin or coagulated serum but is actively saccharolytic, fermenting glucose, lactose, saccharose, salicin, and maltose, but not mannitol. It produces an exotoxin and on inoculation into laboratory animals causes a local oedematous condition.

B. haemolyticus (*Cl. haemolyticum*) has been described as the causal agent of 'bacillary haemoglobinuria' of cattle in America. It has the general morphological characters of the group. Gelatin is liquefied, but the organism

is essentially of the saccharolytic type. Glucose is fermented, but not lactose, saccharose, salicin, or maltose. On blood agar marked hæmolytic is observed and exotoxin can be demonstrated in fluid cultures. This toxin when injected in laboratory animals produces an œdematous condition with blood-staining of the œdema fluid, and sometimes hæmoglobinuria. A condition resembling the natural disease has been reproduced by inoculation of cattle (Vawter and Records)

BACTERIOLOGICAL DIAGNOSIS OF WOUND INFECTION DUE TO ANAEROBES

Samples of the inflammatory exudate are taken with sterile swabs, either on wires (similar to throat swabs) or on 5-inch wooden applicators. Three swabs should be taken from the deep parts of the wound or from the area in which the infection appears to be most active—one for making microscopic films, the others for cultures. Pieces of necrotic tissue from the wound, placed in sterile screw-capped vials or stoppered test-tubes, may also be used for examination.

Microscopic examination. Films are made and stained by Gram's method. From these some indication is obtained of the existence of infection by gas gangrene organisms, when gas gangrene is present. Gram-positive bacilli are used for the microscopic picture, in the case of infection by *B. proteus*. (A similar infection of muscle with streptococci may occur.) The morphological characters and special features of the various gas gangrene anaerobes have been detailed above and in the Table on pp 450-3. It must be recognised, however, that microscopic examination, while providing some diagnostic information, is of strictly limited value.

Cultures. (1) Two blood agar plates are inoculated; one is incubated anaerobically, the other aerobically, for several days. Since *B. proteus*, which is frequently present in wounds, tends to spread over the plates and interfere with the recognition and isolation of other organisms, methods should be followed which inhibit it (p 308). The surface of the anaerobic plate should be well dried to prevent spreading of the growths of certain anaerobes. It should be remembered that many aerobes are also facultative anaerobes and grow quite freely on the anaerobic plate. Careful comparison of the growths on the aerobic and anaerobic plates is therefore necessary.

(2) Two tubes of cooked-meat medium should be inoculated, one of which before incubation is heated at 65° C. for thirty minutes to kill non-sporing organisms. It is also advisable to inoculate a third tube with a broth emulsion of exudate which has been heated for five to ten minutes at 100° C. This procedure is recommended for the isolation of *B. œdematiens*. The tubes are incubated for several days.

(3) Litmus milk contained in a long narrow tube previously boiled and then cooled, is inoculated, and thereafter a layer of melted vaseline is superimposed on the column of milk in the tube.

After forty-eight hours' incubation the colonies on the blood agar plates are examined with the naked eye and by means of a plate-culture microscope, and films are also made from colonies and stained by Gram's method. Pure cultures are obtained by subculturing single well-isolated colonies. The colony characters of the various anaerobes have already been described, see also the Table on pp 450-3. Any suspected anaerobe must be examined in subculture to establish and confirm its anaerobic character, and in this connection

DIFFERENTIAL CHARACTERS OF SPORING ANAEROBIC BACILLI ASSOCIATED WITH GAS GANGRENE, ETC., WHICH ARE PATHOGENIC IN PURE CULTURE FOR GUINEA-PIGS AND MICE AND WHICH FORM EXOTOXINS

FATHOGENIC IN PURE CULTURE FOR GUINEA-PIGS AND MICE AND WHICH FORM EXOTOXINS													
	Special morphological features (1)	Surface colonies (2)	Cooked-meat medium (3)	Milk medium (4)	Liquefaction of		Fermentation of						
					gelatin (5)	coag. serum (6)	Glucose (7)	Lactose (8)	Saccharose (9)	Salicin (10)	Maltose (11)	Mannitol (12)	Glycerol (13)
<i>B. welchii</i>	large, broad, often rectangular bacilli, capsules usually absent in tissues and cultures, non-motile	large, circular, with regular margins (haemolytic on blood agar)	meat red-dened, no digestion	'stormy clot' reaction	+	-	AG	AG	AG	(AG)	AG	-	(AG)
<i>V. septicæ</i>	filamentous forms and 'citron bodies' in tissues	irregular, with spreading out-growths	meat red-dened, no digestion	late coagulation	+	-	AG	AG	-	AG	AG	-	-
<i>B. adermatitidis</i>	resembles <i>B. welchii</i> , but bacilli longer and more pleomorphic, and though not numerous, oval subterminal spores seen in tissues	transparent, tend to become confluent and form spreading film	meat red-dened; no digestion	sometimes late flocculent coagulation	+	-	AG	-	-	-	AG	-	(AG)
<i>B. tetani</i>	'drum-stick' bacilli (spherical spores)	feathery colonies and spreading growth	some digestion and slight putrefactive odour	-	+	(+)	-	-	-	-	-	-	-

<i>B. botulinus</i> type A	large, irregular with filamentous structure and tumefact margins	meat digested and blackened ditto ¹ no digestion	casein precipitated and digested ditto ¹ no digestion	+	+	AG	(AG)	-	AG	AG	-	AG
<i>B. botulinus</i> type B	irregular with iridescent projections	meat red digested	meat red digested	-	(+)	AG	(AG)	-	AG	AG	-	AG
<i>B. botulinus</i> type C	resembles <i>B. subtilis</i> and shows cottony bodies in tissues, but not filaments	meat red digested	meat red digested	+	-	AG	AG	AG	AG	AG	-	AG
<i>B. fallax</i>	resembles <i>B. subtilis</i> but somewhat shorter and thinner	meat red digested	meat red digested	-	-	AG	AG	AG	AG	AG	(AG)	-
<i>B. histolyticus</i>	small, transparent with irregular margins (not strict anaerobe)	blackening and digestion, deposits of tyrosine crystals	coagulation, digestion of clot	+	+	(A)	-	-	(A)	-	-	-
<i>B. difformis</i> (<i>B. mordax</i>)	circular, even edged or irregular, somewhat opaque	blackening and digestion, putrefactive colour	slow coagulation, digestion of clot	+	+	AG	-	-	(AG)	AG	-	AG
<i>B. dysenteriae</i>	irregular with rough surface	no digestion	no coagulation	(+)	-	AG	-	-	AG	-	AG	-
<i>B. cereus</i>	very small, transparent, circular, becoming lobate (not strict anaerobe)	no digestion	no coagulation	-	-	AG	AG	AG	AG	AG	-	-

Symbols within the brackets indicate variability of reaction or varying reports by different observers
 1 Strains vary in digestive action, some resembling type C

A=acid G=gas

— = absence of change or reaction

DIFFERENTIAL CHARACTERS OF VARIOUS SPORING ANAEROBIC BACILLI ASSOCIATED WITH GAS GANGRENE, ETC., WHICH ARE NON-PATHOGENIC IN PURE CULTURE FOR GUINEA-PIGS AND MICE AND DO NOT FORM EXOTOXINS

	Special morphological features	Surface colonies	Cooked-meat medium	Milk medium	Liquefaction of		Fermentation of						
					gelatin	coag serum	Glucose	Lactose	Saccharose	Salicin	Maltose	Mannitol	Glycerol
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
<i>B. tetani</i>	slender bacilli with oval terminal spores	small, transparent, circular, occasional small offshoots (not strict anaerobe)	no digestion	late coagulation	—	—	AG	AG	AG	AG	AG	AG	—
<i>B. butyrans</i>	capsulate in culture, bacilli store glycogen and stain yellow with iodine	small, circular	no digestion	coagulation	—	—	AG	AG	AG	AG	AG	AG	—
<i>B. sporogenes</i>		'medusa head' or with arborescent margins	blackening and digestion; putrefactive odour	precipitation and digestion of casein	+	+	AG	—	—	(AG)	AG	—	(AG)
<i>B. aerofaciens</i>		transparent, circular, may be fimbriate	blackening and digestion; putrefactive odour	coagulation and digestion of clot	+	+	AG	AG	—	(AG)	AG	—	—
<i>B. multiformans</i>	resembles <i>V. septicus</i> ; shows 'citron bodies'	large, with irregular margins, when fully grown raised and opaque	no digestion	coagulation, with some breaking up of clot by gas	—	—	AG	AG	AG	AG	AG	—	AG

it must be remembered that *B. histolyticus* and *B. tertius* are not strict anaerobes. In obtaining pure growths it is further desirable that the first subculture should be re-plated and a subculture again made from a single colony. It may be necessary in some cases to repeat this process further to ensure that the final culture studied is quite pure, since anaerobes have a tendency to form mixed colonies. Confusing results often arise from the study of impure cultures. The cultures are finally examined for their various fermentative reactions (*vide* Table on pp 450-3)

The milk culture is specially useful for the rapid recognition of *B. welchii*, but some strains may fail to give the 'stormy-clot' reaction

The primary cooked-meat cultures are of value if there is difficulty in detecting and isolating anaerobes in plate culture, e.g. in the case of organisms that grow slowly. These cultures should be examined by microscopic film preparations and subcultures made on anaerobic blood agar plates. It must be noted that aerobes which are facultative anaerobes also flourish in this medium. The tubes inoculated with heated material will, of course, represent growths of sporing organisms only.

Certain other cultural methods have also been found of value, especially when anaerobic jars are not available, e.g. shake cultures in agar in tubes or in capillary pipettes stoppered with cotton wool at the wide end and with the capillary end sealed in the flame. Serial decimal dilutions of the exudate are made in broth and each is used to inoculate the melted agar (at 45° C), which is then allowed to solidify in the tubes or pipettes. One or more of the dilutions will yield growths with well separated colonies, which can be transferred for subculture by means of a capillary pipette after cutting the tube

Various reducing substances have also been incorporated in media to produce anaerobiosis, e.g. glucose, ascorbic acid, thioglycollate, and strips of iron

Nagler's reaction is used as a test for identifying toxigenic *B. welchii*.

Equal parts of Fildes' peptic digest broth (or other suitable medium) and sterile human serum are mixed and 0.3 c.c. is placed in each of two small tubes with cotton-wool stoppers. To one tube 0.03 c.c. of a *B. welchii* antitoxin (15 to 30 units) is added. The other tube contains the same organism. They are incubated at 37° C. for sixteen to sixty hours. The tube with a yellow surface 'curd' in the tube containing no antitoxin, and a clear surface in the tube containing antitoxin. The test has also been applied to plate cultures. Agar medium containing 20 per cent human serum and 5 per cent Fildes' peptic digest is poured into a Petri plate. When this has set 0.1 c.c. (50 to 100 units) of *B. welchii* antitoxin (not polyvalent antitoxin) is spread over one half of the surface and allowed to dry. Both halves are then inoculated equally and the plate is incubated under anaerobic conditions. Toxigenic *B. welchii* is indicated by the appearance around the colonies, after eighteen hours or upwards of opaque halos on the medium without antitoxin, whereas the presence of antitoxin prevents halo-formation (see Hayward). *B. bifermentans* produces a weak halo which is inhibited by the antitoxin, but is readily distinguished by other characters.

Animal inoculation tests should also be carried out with the isolated cultures to determine pathogenicity. The pathogenic properties of the various anaerobes have been dealt with earlier in the chapter. Protection tests with specific antitoxins are specially significant in identifying species (e.g. *B. welchii*, *V. septique*, and *B. oedematis*) and also the different types of *B. welchii*. For this purpose mice of 20 grms weight are injected intravenously or intraperitoneally with 50 units of the respective antitoxins and two hours later the culture in question is inoculated into each. There is usually good protection in the animal which received antitoxin for the particular species of anaerobe. The neutralisation effects in the case of the different types of *B. welchii* and their antitoxins are dealt with on p. 439.

CHAPTER XXI

ACTINOMYCES AND ALLIED ORGANISMS

THE term 'actinomycosis' may conveniently be applied to infections caused by a number of allied species in which the organism—variously called *Streptothrix*, *Nocardia*, or *Actinomyces*—forms 'granules' or colonies composed of a mycelium or meshwork of branched filaments, and exhibiting in certain cases radial club-shaped bodies. Actinomycosis occurs in man in common with certain of the domestic animals, though it is more frequent in the latter, especially in cattle, swine, and horses. The lesions are either suppurative or granulomatous—sometimes in the form of tubercle-like nodules—and often they partake of both characters, in cattle there is frequently considerable formation of new tissue. Bollinger first described the organism in 1877, and the botanist Harz applied the name *Actinomyces* or *Ray fungus* owing to the radiate club-shaped structures characteristic of its colonies in the tissues. In 1878 Israel described the same organism in the human subject. In 1902 it was pointed out by Lignières and Spitz that in a common type of actinomycosis in cattle the colonies are formed not by an actinomyces but by a bacillus to which they gave the name of *Actinobacillus*. Madura disease also comes into this group though its etiology, geographical distribution, and pathological features distinguish it from actinomycosis. Other distinct species of mycelial organisms, without the characteristic arrangement, have also been cultivated from isolated cases of disease in the human subject where the lesions resembled more or less closely those of actinomycosis. In several instances the organism has been found to be 'acid-fast', and the actinomyces group is evidently related through intermediate forms with the tubercle bacillus and the other acid-fast bacilli.

ACTINOMYCES

While different biological types or species are included under this generic name, in the typical actinomycosis of man and animals they present similar morphological characters and pathogenic properties, differences between species are mainly in respect of cultural characters. In the following description the general macroscopic and microscopic characters of these organisms will be dealt with first, and also their pathogenic relationships, and the cultural characters of the group will be considered later in relation to the differential features of the accepted types or species.

MICROSCOPIC CHARACTERS OF THE ORGANISM IN LESIONS. The actinomyces grows in the tissues in the form of little round masses or colonies which when fully developed, are easily visible to the naked eye, the largest being about 1 mm. in diameter whilst all sizes below this may be found. When suppuration is present the colonies lie free in the pus; when there is no suppuration they are embedded in the granulation tissue, but are usually surrounded by a zone of softer tissue. They may be seen in the pus by spreading it out in a thin layer. They may be transparent and jelly-like or they may be opaque and of various colours—white, yellow, or greenish. The appearance depends upon their age and also upon their structure, the younger colonies being more or less transparent, the older ones

being usually opaque. They are generally of soft, sometimes tallow-like, consistence, though occasionally in cattle the masses are brittle and consist of calcareous deposit. In the human liver the masses are of semi-translucent specks, greenish yellow. It must be noted that similar granules composed of masses of organisms may occur in the tissues in widely different infections, e.g. botryomycosis in the horse due to a staphylococcus, and sometimes after inoculation with the tubercle bacillus.

MICROSCOPIC CHARACTERS. Three different morphological elements have been recognised, namely, filaments, spores or conidia, and clubs.

The *filaments* are comparatively slender, measuring about 0.6 to 1 μ in diameter, but they are often of considerable length. They are composed of a central protoplasm enclosed by a sheath. The latter, which is most easily made out in the older filaments with granular protoplasm, occasionally con-

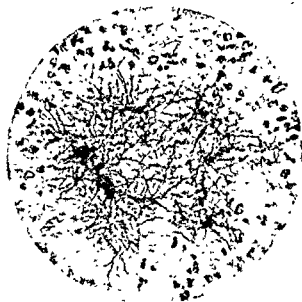


FIG. 121. Actinomycosis of human liver, showing a colony of the parasite composed of a felted mass of filaments surrounded by pus. Section stained by Gram's method and safranin $\times 500$.



FIG. 122. Actinomyces, from a culture on glycerol agar, showing branched filaments. Stained by Gram's method $\times 1,000$.

tains granules of pigment. In the centre of a colony developing in the tissue the filaments interlace with one another, and form an irregular network which may be loose or dense; at the periphery they are often arranged in a somewhat radiating manner, and run outwards in a wavy or even spiral course. They also show true branching, a character which at once distinguishes them from the ordinary bacteria. Between the filaments there is a finely granular or homogeneous ground substance. Most of the colonies at an early stage are chiefly constituted by filaments loosely arranged (Fig. 121); but later, part of the growth may become so dense that its structure cannot be made out. This dense part, starting excentrically, may grow round the colony to form a hollow sphere, from the outer surface of which filaments radiate for a short distance. The filaments usually stain uniformly in the younger colonies and are Gram-negative. In the older colonies the filaments may be segmented, so as to give the appearance of a chain of bacilli or of cocci, though the sheath enclosing them may generally be distinguished. Rod-shaped and spherical forms may also

be seen lying free (Fig 123). In culture some forms of actinomyces develop mainly as rod-shaped structures (resembling diphtheroid bacilli) with only a few branched and filamentous elements

the inoculation strokes can then be examined at intervals during the growth of the organism

Conidia It is questionable whether the common type of actinomyces found in human or animal lesions produces true conidial bodies. Some of the filaments of an actinomyces when growing on a culture medium become segmented into spherical forms, and these structures have been regarded as reproductive conidia and capable of forming new colonies on becoming free from the original growth. They have been reported to possess

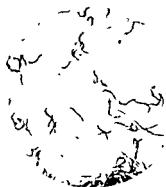


FIG 123 Film of crushed colony of actinomyces from a human case. Stained by Gram's method $\times 1,000$

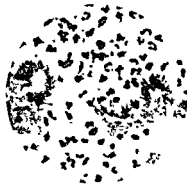


FIG 124 Actinomyces in human kidney, showing clubs radially arranged and surrounded by pus. The filaments had practically disappeared. Section stained with hamatoxylin and fuchsin $\times 500$

somewhat higher powers of resistance than the filaments, though less than the spores of most of the lower bacteria, cultures containing conidia can resist a temperature from five to ten degrees higher than conidium-free cultures (Foulerton). An exposure to 75°C for half an hour is sufficient to kill most actinomycetes or their conidia. The conidia are readily stained by Gram's method.

Clubs These are elongated pear-shaped bodies which are seen at the periphery of the colony, and have often been regarded as enlargements of the sheath around the free extremity of a filament (Figs 124, 125). They are usually homogeneous and structureless in appearance. In the human subject the clubs are often comparatively fragile structures, which are easily broken down and may sometimes be dissolved in water. Sometimes they are well seen when examined in the fresh condition, but in hardened preparations are no longer distinguishable. In specimens stained by Gram's method they are usually Gram-negative, having a darkly-stained filament running for a distance in the centre, and some observers have described a knob-like extremity. In many of the colonies in the human subject the clubs are absent. In cattle, on the other hand, where there are much older colonies, the clubs constitute the most prominent feature, and often form a dense fringe around the colony, staining by Gram's method. Occasionally in very chronic lesions in the human

subject the clubs are also Gram-positive. Intermediate staining reactions have been described in cattle by McFadyean. Clubs are usually somewhat acid-fast and can be stained by the Ziehl-Neelsen method as for the tubercle bacillus if 1 per cent. is substituted for 20 per cent. sulphuric acid. It has been debated whether the club formation represents a defensive reaction on the part of the organism towards the tissues of the host, or whether it is degenerative in nature. In support of the latter view it has been stated that when dead organisms are introduced into the tissues club-formation may occur. But it would appear that two types of club-formation can be recognised; one due to the enlargement of the ends of filaments, as originally described, and another resulting from the deposition around the free ends of the filaments of a lipoid-rich material derived from the tissues (Orskov)



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In the majority of cases of actinomycosis in cattle no filaments can be detected in the colonies, which are composed almost entirely of radially arranged clubs, and it is from such colonies that the *Actinobacillus* is cultivated (*vide infra*). Such conditions are more correctly designated actinobacillosis.

Tissue Lesions. In the human subject the lesions are of a chronic inflammatory type, usually ending in a spreading suppuration. In some cases there is a considerable production of granulation tissue, with only a little softening in the centre, so that the inflammatory lesion feels solid. In most cases, however, and especially in internal organs, suppuration is the outstanding feature, this is associated with abundant growth of the parasite in the filamentous form. In an organ such as the liver, multiple foci of suppuration are seen at the spread appearance, while the pus with the naked eye. In the older parts the abscesses have become confluent, and formed large areas of suppuration.

In cattle the tissue reaction is more of a formative type, there being abundant growth of granulation tissue, which may result in large tumour-like masses, usually of more or less nodular character, and often consisting of well-developed fibrous tissue containing areas of younger formation, in which, however, irregular abscess production may be present. It is not uncommon to find leucocytes or granulation tissue invading the substance of the colony and portions of it may be contained within leucocytes or within giant-cells, which are sometimes present. (A similar invasion of old colonies by leucocytes is sometimes seen in human actinomycosis.) The disease usually remains quite local, or spreads by continuity. It may produce tumour-like masses in the region of the jaw or neck, or it may specially affect the palate or tongue, in the latter producing enlargement and induration, with nodular thickening on the surface—the condition known as 'woody tongue'. Infections in cattle involving the soft tissues and spreading by lymphatics are generally due to the *Actinobacillus* (*vide infra*).

Origin and distribution of lesions. In the human subject the primary lesion is most commonly situated about the face or neck, this indicating that the entrance takes place in the mouth or throat—sometimes in the region of a decayed tooth, the crypts of the tonsil, or some abrasion of the mucous membrane. Swelling and suppuration may then follow in the vicinity and may spread in various directions, the bones often becoming affected. In a considerable number of cases the primary lesion is in some part of the intestine, generally the large intestine, and not infrequently in connection with the appendix. A peculiar affection of the intestine has been described, in which slightly raised plaques are found both in the large and small intestines, these plaques being composed almost exclusively of masses of the actinomyces along with epithelial cells. This, however, is a rare condition. The path of entrance may also be by the respiratory passages, the primary lesion being pulmonary or peribronchial, extensive suppuration in the lungs may result. Infection may, however, occur by the tongue, by the skin surface in any part of the body, and lastly, by the female genital tract, as in a case recorded by Grainger Stewart and Muir, in which both ovaries and both Fallopian tubes were affected.

When the organism has invaded the tissues by any of these channels, secondary or 'metastatic' abscesses may occur in internal organs. The liver is the organ most frequently affected, though abscesses may occur in the lungs, brain (where a primary meningitis may also develop), kidneys, etc. In such cases the spread takes place by the blood stream, and it is possible that leucocytes may be the carriers of the infection, as it is not uncommon to find leucocytes in the neighbourhood of a colony containing small portions of the filaments in their interior.

Source of Infection. At one time the view was held that the source of this

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besides recorded in the human subject a certain number of cases in which there was a history of penetration of a mucous surface by a portion of grain, and in some cases such material has been found embedded in an infected area. It has been shown, however, that the types of actinomyces, *A. israeli* and *A. bovis*, isolated from the majority of cases in man and cattle are strict parasites, incapable of growing as saprophytes outside the body. The common saprophytic actinomycetes which occur in soil and grain grow readily in culture at room temperature under aerobic conditions, whereas the common types of pathogenic actinomyces are micro-aerophilic and cannot grow

They also obtained a similar organism in culture from the disease in cattle. In this species it is the chief causal agent of actinomycosis affecting the bones of the head. Henry cultivated from actinomycotic meningitis an organism which was a strict anaerobe and which exhibited similar characters. Colebrook, in a study of twenty-four strains of human origin in this country, found that twenty-one conformed in their general characters and resembled the Israel and Wolff type, while two others showed slight differences in the characters of their growths. Among a considerable series of strains isolated by us from human cases the great majority have conformed to the Israel and Wolff type.

Serological characters According to Erikson, two groups can be recognised by agglutination reactions: one comprising strains isolated from human cases, the other strains derived from cattle (*A. bovis*).

Experimental inoculation with the organism of Israel and Wolff in various animals, including guinea-pigs and rabbits, has given rise to characteristic colonies.

In guinea-pigs the characteristic nodules usually have not a pronounced growth. In rabbits obtained a nodule as large as a man's fist on inoculation of a cow. Inoculation experiments, however, have yielded irregular results and have often been unsuccessful. Various methods have been recommended with a view to producing experimental infection, e.g. repeated large inocula by the intravenous route, injecting the culture in solid medium, preliminary injection of calcium phosphate, but none of these procedures can be relied on to give uniform results. According to Naeslund's observations the presence of mixed infection plays an important part in conducing to the development of progressive actinomycosis.

Actinomyces graminis² (Bostrom) On agar or glycerol agar at 37° C., growth is generally visible on the third



FIG. 127. Section of a colony of actinomyces from a culture in blood serum, showing the formation of clubs at the periphery. $\times 1,500$

having been dusted with a brownish-yellow powder.

In cultures at an early stage the growth is composed of branching filaments, which stain uniformly (Fig. 122), but later some of the filaments may



FIG. 128. Shake culture of actinomyces in glucose agar, showing the maximum growth at some distance from the surface of the medium.

or fourth day in the form of little transparent drops which gradually enlarge and form rounded projections of a reddish-yellow tint and somewhat transparent appearance, like drops of amber. The colonies tend to remain separate, and even when they become confluent, the nodular character is maintained. They have a tough consistence, being with difficulty broken up, and adhere firmly to the surface of the agar. Older growths often show on the surface a sort of corrugated aspect (Fig. 128), and may sometimes present the appearance of

¹ From illustrations by J. H. Wright.

² This name has been suggested by Topley and Wilson.

pus made at random in the usual way and suitably stained may in certain cases reveal the organism, it is essential that actual granules or colonies should be examined. In the case of sections of the tissues, Gram's method with a counterstain yields excellent staining of the mycelium. In cattle the clubs are strikingly demonstrated by staining with carbol-fuchsin and then decolorising with picric-alcohol; or the preparation may be decolorised with 1 per cent. sulphuric acid and then counterstained with methylene blue.

Cultures should be made both under aerobic and anaerobic conditions on glucose-agar plates and in shake cultures. Magnusson recommends keeping the cultures in an atmosphere of carbon dioxide. The medium should be inoculated directly with whole colonies from the pus. Owing to the slow growth of the actinomyces, however, the obtaining of pure cultures is somewhat difficult, unless the pus is free from contamination with other organisms. When other organisms are present the granules should be picked out with a loop and added to sterile saline in a tube, then washed with several changes of saline. They are then shaken in absolute alcohol for half a minute, washed again in three changes of saline, and finally transferred to the culture medium. M. Gordon has recommended for primary cultures from pus, the use of blood broth (in tubes) under a layer of sterile paraffin to secure anaerobiosis if necessary. In this medium the organism grows well in the form of granules. If a mixed growth results with other organisms present, the colonies in the medium, after washing with sterile saline, are transferred to plates, and in this way a pure growth can be obtained. In infections with *Actinobacillus* the serum contains agglutinins for the organism and the agglutination test may be of some value for diagnostic purposes.

LEPTOTHRIX

The designation, leptothrix, has been applied in medical bacteriology to those filamentous organisms which generally resemble the actinomycetes, but differ from the latter in the absence of branching (Figs 4, 12). A leptothrix type is a common inhabitant of the mouth and has been spoken of as *L. butcalis*, it may be found in the tartar deposits on the teeth. This organism seems to flourish especially in the pockets between the gums and teeth in pyorrhœa alveolaris and its growth has been regarded as a factor in tartar formation which is so marked in pyorrhœa (Bulleid). A leptothrix may also occur in the tonsillar crypts. Concretions in the lachrymal duct have been found to be largely composed of growths of this type of organism. We have observed a 'mycosis' of the tongue produced by a leptothrix. Leptothrices have also been recorded in inflammatory and suppurative conditions in the region of the mouth and throat, but the pathogenicity of these organisms seems to be relatively weak. Gifford has described a leptothrix in a case of recurrent conjunctivitis. The leptothrix group has not been studied in sufficient detail from the biological standpoint to merit any classification. The organisms are usually observed in the form of elongated unbranched filaments which are typically Gram-positive. Shorter forms may also be noted similar to large bacilli. They are usually non-motile. Both aerobic and anaerobic strains have been described. Cultures on gelatin may produce liquefaction. Pigment-producing types have been noted. Free spores have also been observed (See Gifford.)

CHAPTER XXII

BACILLUS (OR ACTINOMYCES) NECROPHORUS: BACTEROIDES:
BACILLUS FUSIFORMIS: BACTERIUM PNEUMOSINTES:
STREPTOBACILLUS MONILIFORMIS. BACTERIUM MONO-
CYTOGENES· ERYSIPELOTHRIX· ORGANISM OF BOVINE
PLEURO-PNEUMONIA AND ALLIED ORGANISMS. BAC-
TERIUM TULARENSE

BACILLUS (OR ACTINOMYCES) NECROPHORUS

THIS organism has been found associated with various necrotic and suppurative lesions in animals ('necrobacillosis'). It was described as the cause of 'diphtheria' of calves by Schmorl and others, and occurs in liver abscesses ('bacillary necrosis') in various domesticated animals. Other conditions in which it is found are gangrenous dermatitis of horses, 'foot-rot' of sheep, and necrotic stomatitis of pigs. In the human subject it has been reported occasionally in suppurative lesions, including sloughing septic wounds, but has been isolated from the mouth and intestine of healthy persons and also from the vagina.

The organism is pleomorphic, varying from coccoid forms and slightly curved rods to long wavy filaments arranged in felted masses. These may attain a length of even 100 μ . Sometimes thickenings are present in the course of the filaments or at their ends. Branching is not a feature, but has been described by some observers. The organism is Gram-negative and non-acid-fast. When stained it may exhibit a beaded appearance.

Cultures are obtained under anaerobic conditions on serum agar or blood agar at an optimum temperature of 31° to 36° C. The colonies are small, circular, and transparent with dentate or fimbriate borders, but sometimes large colonies develop. Cultures have a characteristic 'cheese-like' or foul smell. This is particularly noticeable when the culture is grown in milk. The organism is non-proteolytic, but produces indole. Acid and gas are produced from glucose and maltose, but not from lactose or mannitol.

Rabbits and mice are very susceptible to experimental inoculation, *e.g.* subcutaneously. A marked local necrotic lesion develops and they often die. The organism produces apparently a necrotising endotoxin. Strains derived from man seem to be less pathogenic to experimental animals than those isolated from animal disease.

A bacteriological diagnosis of the infection can be established by microscopic examination of stained films made from the necrotic tissue, followed by cultivation of the organism, but direct cultures are often difficult to obtain owing to admixture of other bacteria. This difficulty may be overcome by inoculating rabbits or mice with material from the lesion and then isolating the organism on serum agar under anaerobic conditions from the lesion produced in the inoculated animal.

The taxonomy of this organism presents difficulty. While some have classified it with *Actinomyces*, others have suggested that it is more allied to *B. fusiformis*. *B. funduliformis* is a somewhat similar organism as regards biological characters and pathogenicity, and is included by American systematists in the genus *Bacteroides* (*vide infra*). The classification and relationships of these organisms require further investigation.

BACTEROIDES

Reference has been made in Chapter I to the general characters of organisms classified in this genus by American systematists. The organism originally designated *Bacillus fragilis* (*Bacteroides fragilis*) may be taken as a representative species. It has been isolated from acute appendicitis, suppurative lesions of the urinary system, pulmonary gangrene, and from cases of septicaemia. It occurs in the form of small, non-motile, non-sporing, Gram-negative bacilli, which may show polar staining. It is an obligate anaerobe. Cultures can be obtained on ordinary media at 37° C., but growth is not abundant. The colonies are small, greyish, and irregular in outline. Gelatin is not liquefied and indole is not produced. Glucose, galactose, levulose, saccharose, and maltose are fermented with acid production and gas; and some strains also ferment lactose. Sulphuretted hydrogen is not formed.

On experimental inoculation cultures have been found to be pathogenic, producing subcutaneous suppuration in rabbits and other animals, and sometimes extensive necrosis of tissue.

The various members of the genus appear to be inhabitants of the intestinal tract of man and mammals and some at least possess potentially pathogenic properties. Many of the species or types described have not been fully investigated and the taxonomy of the group is still doubtful. Its relationships to *B. fusiformis*, *Dialister*, and *B. necrophorus* have also to be considered, and all these organisms might be grouped together; this requires further study (see Prévot, Eggerth and Gagnon).

BACILLUS FUSIFORMIS (*FUSOBACTERIUM*, PLAULT-VINCENTI)

Babés in 1884 described organisms of this type in a diphtheria-like affection of the fauces, and since that time the presence of similar organisms has been noted in necrotic inflammatory conditions, ulcerative gingivitis and stomatitis, noma, gangrenous balanitis, and like affections. They have also been found in pulmonary lesions and in abscesses in other parts of the body; in these the pus is very foul-smelling. The association of fusiform bacilli with a form of angina has been specially recognised since the work of Plaut and of Vincent, and this condition often goes now under the name of 'Vincent's angina'. He recognised two forms of the affection—(a) a diphtheroid type, characterised by the formation of a firm yellowish-white false membrane, very like that of diphtheria, associated with only superficial ulceration; and (b) an ulcerative type, where the membrane is soft, greyish, and foul-smelling, attended with ulceration and surrounding cedema. In the former type fusiform bacilli may be present alone, in the latter, which is distinctly the commoner, there are also spirochaetes. The fusiform bacilli are straight or slightly curved, and are tapered at their extremities, measuring 5 to 14 μ in length, and less than 1 μ in thickness. The central portion often stains less deeply than the extremities, and not infrequently shows unstained points and granules (Fig. 131). The organisms are non-motile. They stain fairly deeply with Löffler's methylene blue or with weak carbol-fuchsin, and are Gram-negative. The spirochaetes are long delicate organisms showing several irregular curves, and are motile; in appearance they resemble *Borrelia refringens* and similar organisms found in gangrenous conditions. They stain less deeply than the bacilli. Sometimes they are numerous, sometimes scanty. They have been designated *Borrelia vincenti*. In a section through the false membrane, when stained with methylene blue or thionin,

there is usually to be seen a darkly stained band, a short distance below the surface, which is due to the presence of large masses of the fusiform bacilli closely packed together; neither they nor the spirochaetes appear to pass deeply into the tissues. It is also to be noted that fusiform bacilli are sometimes present in the secretions of the mouth in normal conditions, and may occur in increased numbers in true diphtheria. Vincent's observations have been confirmed by others, and there is no doubt that fusiform bacilli, of which there may be several types, are associated with various spreading necrotic lesions. Ulcerative gingivitis and stomatitis have been found to be associated with the presence of the same organisms, and in some cases these lesions precede the infection of the fauces. It would be advisable to apply the term 'Vincent's infection', so as to include all the conditions produced by *B. fusiformis* and *Bor. vincenti*. In phagedenic lesions of the geni-



FIG. 131. Film preparation from a case of Vincent's angina, showing fusiform bacilli and spirochaetes stained with dilute carbol-fuchsin $\times 1,000$.

taha, fusiform bacilli are usually present, with or without spirochaetes, though in our experience they are as a rule of smaller size than those met with in the throat. Vincent's infection has also been found in certain forms of tropical ulcer (*ulcus tropicum*). Cultures of fusiform bacilli have been obtained by Ellermann, Weaver and Tunncliffe, Smith, and others. They grow only under anaerobic conditions, and the best media are those consisting of a mixture of serum or blood and agar, e.g. 10 per cent blood agar. The optimum temperature is 37° C. and growth does not occur at room temperature. The organisms form small rounded colonies of whitish or yellowish colour, somewhat like those of a streptococcus, but rather felted in appearance on the surface. Older colonies may show marginal projections. In cultures, especially in fluid media, undulating filamentous forms may be found, but there does not appear to be sufficient evidence that the fusiform bacilli and the spirochaetes found in Vincent's angina are stages in the development of the same organism, as maintained by Tunncliffe. Krumwiede and Pratt, for instance, found that the filamentous forms were not true spirochaetes, but merely represented variations in morphology. These observers studied the fermentative properties of *B. fusiformis* and found that it fermented various

sugars without the formation of gas; they distinguished two types, one fermenting saccharose, the other not. Knorr has distinguished three types.

Injections of pure cultures in animals sometimes produce suppuration but never necrosis (Ellermann).

It is probable that there exists a group of these organisms; but difficulties in their isolation and cultivation have hindered exact study of them. Their relationships to such organisms as *Bacillus necrophorus* and the so-called *Bacteroides* group also require further study.

BACTERIUM PNEUMOSINTES (*DIALISTER PNEUMOSINTES*)

In 1921 Olitsky and Gates described a supposed filter-passing agent in the naso-pharynx of cases of influenza, which could only be demonstrated in the earliest stage of the illness and was absent after thirty-six hours from the onset. When inoculated intratracheally in rabbits it produced fever, leucopenia, haemorrhages in the lung with oedema and emphysema. Also, it apparently underwent increase in the lungs and could be transmitted through animals in series. This agent could be passed through Berkefeld V and X filters, and withstood 50 per cent. glycerol for long periods. It was identified as an exceedingly minute, non-motile, coccid or bacilloid structure measuring 0.15 to 0.3 μ in length and designated *Bacterium pneumosintes*. Cultures from filtrates were obtained in Smith-Noguchi medium, and, when inoculated intratracheally in rabbits, reproduced the effects already described as following the inoculation of filtrates of the naso-pharyngeal secretion. The condition produced in animals by the filterable organism was regarded as analogous to the primary condition in epidemic influenza. The occurrence of a similar organism in influenza has been noted by others in different parts of the world (Gordon, Lister, and others). Later, Olitsky and Gates, Garrod and others, showed that similar anaerobic filter-passing Gram-negative organisms may occur normally in the throat secretions; and it is now accepted that *Bact. pneumosintes* has no relationship to epidemic influenza. It is doubtful if such organisms have any pathogenic rôle.

While the characteristic morphological appearances are those given above, after continuous artificial culture the bacillary form becomes more marked, the length of the organism increasing to 0.5 or 1 μ . It may occur singly, in pairs, or even in short chains. It is best demonstrated in culture by staining with polychrome methylene blue. It can also be stained, however, with simple stains, and is Gram-negative. It is a strict anaerobe. When first grown in Smith-Noguchi medium a cloudiness forms in the medium, starting after three to four days at 37° C., at the foot of the tube round the tissue fragment. Colony growths can be obtained on blood agar. The colonies are very small and transparent and take several days to appear. Growths can also be obtained on glycerol-egg medium and Löffler's serum.

The relationships of this organism to other bacteria are unknown. American systematists have classified it in a separate genus, designated *Dialister*.

STREPTOBACILLUS MONILIFORMIS

This organism was isolated by Levaditi and his co-workers from the blood in cases of erythema multiforme and was described in America by Parker and Hudson as the causal agent of a condition designated by them 'Haverhill fever—*Erythema arthriticum epidemicum*'—an acute or subacute febrile illness characterised by multiple arthritis and an erythematous skin eruption.

It has also been reported in epizootics in mice (Levaditi *et al* ; Mackie *et al* ; and Strangeways) and has been found in the nasopharynx of rats (Strangeways). This organism is responsible for a proportion of cases of 'rat-bite fever', where the wound has been infected from the secretions of the animal's mouth. The resulting disease resembles Haverhill fever (see Brown and Nunemaker). (It should be noted that another type of rat-bite fever is due to *Spirillum minus*—*vide p* 509. Although the two forms of illness are very similar, the spirillar infection tends to have a longer incubation, and arthritis is not a feature, also this disease responds readily to arsenical drugs.)

In mice the organism produces a general infection and multiple arthritis. The joints of the feet and vertebrae are frequently affected and swellings of the feet, legs, and tail are noticeable. Paralysis of the hind legs may occur, apparently as a result of vertebral lesions. *Post mortem* there may be



FIG. 132 *Streptobacillus moniliformis*. Film from culture showing filamentous forms with fusiform expansions. Stained with dilute carbol-fuchsin. $\times 1,000$

enlargement of the spleen, which shows areas of necrosis. The liver is sometimes similarly affected. Conjunctivitis and enlargement of lymph nodes are also common features.

MICROSCOPIC CHARACTERS. The organism is exceedingly pleomorphic, occurring as non-motile, short Gram-negative bacilli (1 to 3 μ by 0.3 to 0.1 μ) or as elongated filaments which may show fusiform or globular expansions (Fig. 132). These are most frequently seen in recently isolated cultures. Some observers have described branching of the filaments and it is possible that streptothrix-like forms reported by Schottmüller and others in cases of rat bite fever have been strains of this organism. While in culture the filamentous forms may suggest mycelium formation there is no true branching (van Rooyen).

CULTURAL CHARACTERS. A high concentration of blood or serum is necessary for maximum growth on culture medium. Löffler's serum is specially suitable. Growth occurs aerobically and also under anaerobic conditions and best at 37°C. No growth occurs at room temperature. The colonies are at first pin point in size and do not as a rule exceed 1 mm. in diameter. They are circular, transparent, slightly convex and colourless.

Cultures die within two or three days and even after continued cultivation viability is feeble. For obtaining blood cultures the most important factor seems to be a high proportion of protein in the mixture, e.g. 1 volume of the unclotted blood added to 4 to 9 volumes of broth (if the illness has lasted over a week it is advisable, in case antibodies have developed, to centrifuge heparinated blood from the patient and to add the sediment to broth *plus* serum—animal or human). On incubation colonies of the organism are seen as minute fluffy balls resting on the sedimented red cells. Strains appear to be homogeneous in their serological characters.

Klieneberger has described in cultures of *Streptobacillus moniliformis* a symbiont which resembles the organism of pleuro-pneumonia. The symbiont, observed by means of impression preparations of colonies, shows no bacillary structures, but occurs in the form of filterable granules, filaments, and other structural elements similar to those of the pleuro-pneumonia organism (*vide infra*). Klieneberger has also reported the separation of the symbiont from cultures of *Streptobacillus moniliformis*. Van Rooyen, who has studied the morphology of this organism in impression preparations, has been unable to confirm the existence of an independent colonial system as described by Klieneberger, and has drawn attention to the occurrence in the organism of characteristic refractile granules which may also occur extracellularly. He states that they show a close similarity to the granular phase of the symbiont described by Klieneberger. It is difficult to interpret these findings. The frequent occurrence of the 'pleuro-pneumonia' *Streptobacillus moniliformis* might suggest the latter. On the other hand, its isolation point to its being a separate entity. It is noteworthy that both organisms occur in the pharynx of rats.

EXPERIMENTAL INOCULATION. Mice can be infected experimentally by inoculation with cultures, but different races of these animals vary considerably in their susceptibility both to the spontaneous and the induced infection. The Simpson-March albino strain is specially susceptible. The ordinary hybrid black-coated stock mice are highly resistant. Successful inoculation leads to a rapidly lethal general infection or a subacute disease with localised lesions in bones and joints, as in the spontaneous infection (*vide supra*).

BACTERIUM MONOCYTOGENES (*LISTERIA MONOCYTOGENES*)

This organism was described by Murray, Webb and Swann in a disease of rabbits in which an excess of large mononuclear cells in the blood was a striking feature. It has also been reported in various animals, e.g. gerbils, sheep, and in meningeal infections in man (Burn, Wright and MacGregor). The same organism has been found in cases presenting the clinical features of infectious mononucleosis; this condition has been generally regarded, however, as a virus disease. The organism when inoculated in rabbits and guinea-pigs, produces a monocytosis like that of the natural infection and may localise in the myocardium, liver, or spleen, producing necrotic lesions and in the meninges with associated inflammation. Mice are highly susceptible to experimental inoculation. The microscopic characters are those of a Gram-positive, non-sporing bacillus with rounded ends, varying in length from 2 to 3 μ and about 0.5 μ broad. The bacilli may be disposed in pairs end-to-end or lying at an angle to one another, they may show a considerable degree of pleomorphism. No branching is observed, but filamentous forms have been seen. The organism is motile and a single terminal flagellum

has been described. Motility may not be pronounced and is best seen by dark-ground illumination. The staining may be either uniform or irregular, the organism in the latter case having a beaded appearance. Cultures can be obtained under aerobic conditions at 37° C. on ordinary media, but growth is better on a medium containing blood or liver extract. The colonies are small and transparent. Old cultures have a tendency to become mucoid. The organism grows on a blood-tellurite medium producing minute, dark-brown glistening colonies. Glucose is fermented and to a slight extent various other carbohydrates. Indole is not produced. Gelatin is not liquefied. The antigenic characters of *Bact. monocytogenes* have been studied by Paterson. By agglutination and agglutinin-absorption tests he has recognised four types differing in their somatic and flagellar antigens. He has found human strains in all four types, strains from rodents in one type and from ruminants in another, strains from poultry have been found in two types. The source of infection in human cases remains obscure, so far it has not been possible to trace these definitely to animals. It has been suggested from clinical findings that the organism may reach the meninges via the middle ear. The biological relationships of this organism are doubtful. Some observers have placed it in a separate genus (*Listerella* or *Listeria*), but it may be related either to the *Erysipelothrix* or diphtheroid group.

ERYSIPELOTHRIX

An organism of this group (*Erysipelothrix* or *Bacillus rhusiopathiae*) is responsible for a disease of pigs known as 'swine erysipelas'. Cases of human infection ('erysipeloid') by the organism have been observed, the skin of the forearm or hand usually being affected, e.g. in abattoir workers. Septicæmia may also occur. The organism has been found in the tonsils, intestine, and faeces of apparently healthy pigs. Though potentially pathogenic, it is apparently a normal inhabitant of the alimentary tract of these animals. It may exist in dung heaps, sewage, and soil and has been found in fish. In the acute form of the disease there is a general infection, and the organism can be demonstrated microscopically in the blood, especially inside leucocytes. A characteristic of the disease is the occurrence of multiple diamond-shaped patches of congestion, and often hæmorrhages, in the skin—hence the designation 'erysipelas'. In chronic cases there may be a 'verruccose' endocarditis. In some cases arthritis occurs. The organism is a slender non-motile Gram-positive bacillus (1 to 2 μ by 0.2 to 0.4 μ), but may develop longer filaments and show true branching. It is a micro-aerophile organism,

of solid media are very small, delicate, and transparent, and rarely exceed half a millimetre in diameter. Cultures may show also a rough colony variant, which is larger than the smooth form, has a granular structure and fimbriate border. This variant is less virulent than the smooth form. The disease has been reproduced in pigs by inoculation of cultures. Inoculation of mice produces a general infection which is fatal within a few days. Pigeons are also highly susceptible. Subcutaneous inoculation of rabbits leads to a diffuse inflammation with œdema. Animals can be protected against the disease by administration of an immune serum followed by inoculation with a virulent culture. The disease can be treated with immune serum and this should be employed also in human cases. Multiple serological groups of the

organism have been recognised and differentiated by agglutinin-absorption reactions (Gledhill). The bacteriological diagnosis of cases is established by the microscopic demonstration of the organism in the lesions or blood and its cultivation from these sources. Confirmatory evidence is obtained by inoculation of mice or pigeons either with pathological material or pure cultures. The agglutination test with the serum of a suspected case may also indicate the presence of the infection. A similar organism (*E. muriseptica*) has been found in a septicæmic disease of mice.

THE ORGANISM OF BOVINE PLEURO-PNEUMONIA

The organism of bovine pleuro-pneumonia is a link between the pathogenic bacterium and an ordinary culture medium such as serum, to which 10 per cent. ox or horse serum has been added, as Nocard and Roux showed, and in such cultures it appears as microscopically visible bodies of various sizes and shapes stainable by the usual methods. On the other hand it exists in a form capable of passing through filters which hold back the ordinary bacteria. Growth is visible after several days' incubation at 37° C., a slight turbidity develops in the medium and becomes more pronounced in the fluid seen in the fluid may be got in the transparent colonies, which after four to five days at 37° C. have a diameter of about 0.2 mm., with a granular brownish surface and opaque centre. Finally the colony is 1 mm. in size; it is whitish, umbonate, and can be detached from the medium only with difficulty. There is no growth below 30° C. Acid is produced from a number of fermentable substances. Cultures die in a few weeks, the development of the fermentation of glucose, hastening in successive subcultures the virulence gradually becomes attenuated. Heating for one hour at 58° C. kills cultures.

Borrel with others recognised that this organism showed great pleomorphism and that certain forms appeared in succession in cultures. It is Gram-negative at all stages and requires for its satisfactory demonstration fairly intense staining, e.g. with warm Giemsa's solution. The tenacious character of the colonies, due to a mucoid matrix which is formed, has hindered the elucidation of the microscopic appearances, and artefacts in microscopic preparations from cultures have also caused confusion. Ledingham used the method of impression-preparations in order to study the growths (Fig. 133). Starting from the filterable elements, which are regarded as consisting of condensed but plastic masses of free protoplasm essentially similar to those composing non-filterable elements, filaments grew out which showed branching. Then the filaments, either at their ends or in their course, produced beads of great variety in size and shape and with intense affinity for chromatin stains. From these there arose rings, spheres, vibrios, filaments, and other forms seen in cultures at the height of growth. Around the deeply staining material which with Giemsa's solution assumed the purple colour of chromatin, a pale blue-stained sheath was seen. Ledingham considered that the organism shows close affinities with the actinomycetes. Earlier observers gave the organism the designation *Asterococcus mycoides*.

The developmental cycle has also been studied by Tang and his co-workers, whose results may be summarised as follows: there is an elementary granular phase consisting of coccoid or cocco-bacillary forms, 0.15 to 0.4 μ in diameter, these granules develop into spheroids, 0.4 to 0.8 μ in diameter and from the

latter peripheral buds are formed, the buds separate from the original structure, but remain attached by a delicate filament, protoplasmic condensations form in these filaments and from them new filaments develop, thus, the

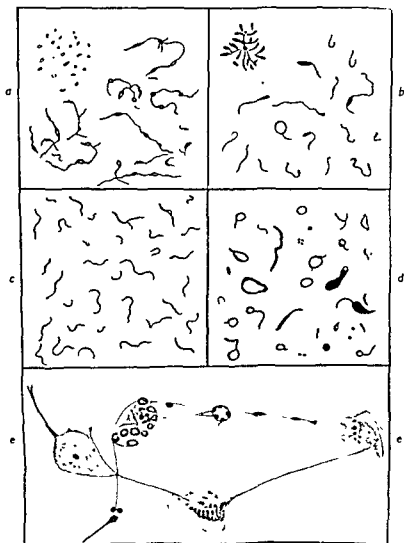


FIG. 131 Growth Phases of the Organism of Pleuro pneumonia a, filterable spore-like initial corpuscles and filamentous branched

(Modified from Ledingham)

filaments grow and branch to form an interlacing mycelium later the protoplasm of the filaments becomes condensed so as to form chains of coccus like bodies, finally the chains disintegrate into the elementary granular bodies. Kheneberger and Smiles have described a simpler process of reproduction, the elementary granules develop into spheroids, which become irregular or filamentous and undergo segmentation into multiple derivatives, within

such segments deeply-staining bodies like nuclei may appear and later subdivide inside a limiting membrane, this being followed by a subdivision of the protoplasm, so that a number of merozoite-like derivatives result as in the schizogony of the malaria parasite; these finally become free and their cytoplasm is lost, the residue constituting the elementary granule. The descriptions of the morphology and reproduction of the organism given by different observers leave considerable doubt as to its true characters, classification, and biological relationships, and these require further investigation.

Experimental inoculation. Subcutaneous injection in cattle with exudate from the natural disease or with a virulent culture produces a local inflammatory lesion, and death may ensue. At autopsy an abundant serous exudate is observed in the interstitial tissue of the lungs with some exudate also in the pleura. Daubney has recorded the reproduction of the typical disease by intravenous inoculation of exudate or culture in agar which is arrested in the lungs and forms initial foci from which the pleuro-pneumonic condition develops. Laboratory animals are not susceptible to experimental inoculation.

Immunity. Immunisation against the disease has been achieved by inoculating exudate or a living culture of the organism into the point of the tail; this does not bring about the typical infection and produces an 'immunity lasting for about a year. Convalescent serum has been used to produce passive immunity. Specific antibodies can be demonstrated in immune serum. Serological differences have been found among strains.

OTHER ORGANISMS RESEMBLING THAT OF PLEURO-PNEUMONIA

Agalactia, a contagious disease of sheep and goats, characterised by inflammation of the udder, eyes, and joints, is due to an organism similar to that of pleuro-pneumonia, which can be isolated from the affected joints and related lymph glands. The disease has been reproduced in goats by inoculation of cultures. A solid immunity follows recovery.

LI as a symbiont of the same type
pure culture from the lung of a rat. The question whether LI is an independent species or a variant of the streptobacillus has already been discussed. The latter organism occurs normally in the pharynx of rats. Other types have been isolated from rats (bronchiectatic lesions, submaxillary gland, and polyarthritis) and from mice (polyarthritis, the brain in 'rolling disease', conjunctiva, and respiratory tract). Strains have been described in dogs and guinea-pigs. Saprophytic strains have also been described, e.g. in sewage, soil, and manure (Laidlaw and Elford, Seifriz).

Dienes has demonstrated organisms of the group in the cervix uteri of patients with pelvic infection, and they have been described by Salaman in inflammatory exudates in the genital tract of both men and women, often associated with gonorrhoea and *Trichomonas* vaginitis but sometimes apart from any pathological condition.

The association of such organisms with polyarthritis of rats and mice has raised the question of their possible relationship to acute rheumatic disease in man, but there is no evidence so far that they have any relationship to rheumatic lesions.

For detailed information regarding this group and the various types described the following authors should be consulted: Klieneberger; Dienes; Findlay *et al.*, Laidlaw and Elford; Sabin.

BACTERIUM TULARENSE (*PASTEURELLA TULARENSIS*) OF TULARÆMIA (DEER-FLY FEVER, OR OHARA'S DISEASE)

This organism was first described by McCoy and Chapin in a plague-like disease of ground-squirrels in California. The infection also occurs in other wild rodents, e.g. jack-rabbits, rabbits, hares, lemmings, field-mice, etc., and has been found in sheep, calves, and birds. The organism is a very minute bacilloid or coccoid body measuring $0.3\ \mu$ to $0.7\ \mu$ in its longest diameter. It stains Gram-negatively and in films from animal lesions often presents the appearance of a capsule. It can be demonstrated microscopically in the spleen of infected animals where it may be present in enormous numbers. Artificial

has proved very suitable: meat extract, 1 per cent; peptone, 1.5 per cent; agar, and 0.5 per cent NaCl, adjusted to a pH of 7.3, before use. 0.1 per cent cystine and 1 per cent glucose are added, and the mixture is steamed for fifteen minutes, then cooled to 45° to 50° C. and 5 to 10 per cent rabbit blood or 5 per cent sterile human serum added (Francis). Growth may also be obtained either on blood agar or serum-glucose-agar with a piece of sterile rabbit's spleen. The colonies grow slowly and are small, translucent, and slightly mucoid. The thermal death point is between 55° C. and 60° C.

According to Hesselbrock and Foshay the morphology of *Bact. tularense* is more complex than that which has been generally observed; they describe filamentous forms and minute reproductive units in addition to the coccoid and bacilloid forms; they regard the process of budding as the main reproductive mechanism and consider that the organism has a relationship to that of bovine pleuro-pneumonia.

The infection, spoken of as tularæmia, is transmissible to man, and a considerable number of cases have now been recorded in North America and also in Norway, Russia, and Japan. The disease is characterised by an indolent primary lesion on the skin, with persistent enlargement of the related lymph-glands, which tend to break down, as well as constitutional disturbance, but fever may be the only symptom. In fatal cases pneumonic patches are present in the lung in about 70 per cent. The exudate is usually mononuclear and necrosis is common. Similar focal lesions in the liver are found in about half of the cases. Certain of the cases have resulted from contact with infected animals, e.g. through handling diseased rabbits and other rodents. Cases may arise also from the bite of blood-sucking flies and ticks, and strains of *Bact. tularense* have been isolated from ticks. Water polluted from infected rodents may also be a source of infection either through the alimentary tract or by the skin; thus the organism, as shown experimentally in animals, may pass through the intact skin. Infection may also occur by the conjunctiva. It is specially remarkable how readily laboratory workers may become infected by handling infective material and artificial cultures, and cases of such laboratory infection have occurred both in America and in this country, leading to a somewhat protracted period of invalidism. Artificial cultures apparently retain a remarkably high degree of virulence and infectivity. Laboratory animals, e.g. guinea-pigs on intraperitoneal inoculation, can be infected experimentally from cultures or infected blood, etc., and at autopsy the principal pathological changes are multiple necrotic foci in the spleen, liver, and lymph nodes. The pathological picture varies considerably in different organs and in different species. According to

spirochæte in the lesions. The cause of syphilis is the organism discovered by Schaudinn and Hoffmann in 1903 and called by them the *Spirochæta pallida*, now known as the *Treponema pallidum*. They described its characters and its occurrence in syphilitic lesions, and their observations have been fully confirmed. Recognition of this organism was long delayed owing both to the difficulty in detecting it in the fresh state on examination by transmitted light and to its weak affinity for stains. Before the demonstration of the spirochæte the successful transmission of the infection to experimental animals by Metchnikoff and Roux (1903-03) provided a means of systematic study of the disease under controlled conditions. Nevertheless many problems still require elucidation.

MICROSCOPIC CHARACTERS. *Tr. pallidum* is a minute organism of spiral shape, showing usually from six to fourteen curves, though longer forms are met with; the curves are small (each measuring approximately 1μ in depth and breadth), comparatively sharp, and regular (Figs. 131, 135). It measures 6 to 14μ in length, while it is extremely

thin, its thickness being only 0.25μ . In a fresh specimen, say, in exudate from a chancre, the organism shows active movements, which are of three kinds—rotation about the long axis, gliding movements to and fro, and movements of flexion of the whole body; there is little actual locomotion, and a spirochæte will often remain in the same field for a long time, although the organisms as seen in the testicular lesion of experimentally infected rabbits may show active movements of progression. Movements may cease in a few hours; on the other hand in sealed capillary tubes or wet preparations kept at room temperature



FIG. 131. Preparation from exudate of hard chancre showing *Treponema pallidum* Giemsa's stain $\times 1,000$

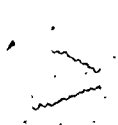


FIG. 135. Film preparation from exudate of hard chancre showing *Treponema pallidum* Becker's stain $\times 1,500$



FIG. 136. Film preparation from Luesitis showing *Borrelia refringens*. Becker's stain. $\times 1,700$

motility may persist for several weeks (see Lumsden). Each end consists of a straight terminal filament considered by some not to be a true flagellum; although appearances obtained with the electron microscope have been interpreted as lateral flagella. In fresh specimens and in dried films (Figs. 131, 133) the regularity of the spirals is well maintained, though in the latter there is sometimes distortion or drawing out of a spiral. When stained by any of the Romanowsky solutions, e.g. Giemsa's or Leishman's, it appears of a faint pink tint; also it is thinner than when stained by Becker's method. Division occurs at right angles to the long axis.

Variations in morphology. Noguchi, on studying different strains of the *Tr. pallidum* in cultures, found that they varied in thickness, and he distinguished thick, thin, and intermediate types. He found that they differed also in their pathogenic action, the thick forms on injection into the testicle of a rabbit causing nodular lesions of cartilaginous hardness, while the thin forms produced a diffuse indurative lesion; the difficulty of obtaining

have postulated a cycle of development of *Tr. pallidum* in order to explain the high infectivity of tissues in which no spirochaetes can be detected on microscopic examination, e.g. the lymph glands of inoculated rabbits after the infection has become latent. They conclude also that a similar cycle occurs on experimental inoculation of the central nervous system with *Tr. pallidum*. One stage in the cycle is believed to be ultramicroscopic. While these views would explain certain difficulties in connection with the pathology of neurosyphilis, there is no definite evidence supporting them (*vide infra*).

Distribution of Tr. pallidum in the tissues. In the primary sore in the stage of active ulceration, the organism has been found in a very large majority of cases; but the number of spirochaetes decreases as healing sets in. It can be demonstrated in the lymphatic glands related to the site of the chancre. It has also been obtained regularly in the papular and roseolar eruptions, in condylomata and mucous patches—in fact, one may say generally, in all the secondary lesions.

Also, during life in secondary syphilis on a few occasions it has been detected in the blood and it has been demonstrated in the spleen. In the congenital form of the disease the organism may be present in large numbers widely distributed, as was first shown by Buschke and Fischer, and by Levaditi. In the pemphigoid bullae, in the blood, in the internal organs—the liver, suprarenals, lungs, spleen, and even in the heart—its detection may

(Fig 137) In the large numbers present in the interior of the more highly specialised cells, for example, liver cells. They also abound sometimes on mucous surfaces, e.g. of the bladder and intestine in cases of congenital syphilis. The enormous numbers of this organism which may be present in a well-preserved condition in macerated foetuses render it probable that the organism may multiply in the dead tissues under anaerobic conditions. It is



FIG. 137. Section of liver from a case of congenital syphilis, showing numerous examples of *Treponema pallidum*. Levaditi's method $\times 1,000$.

in accordance with this view, as they gave negative results. More prolonged search has shown, however, that the organism may occur in tertiary lesions also. It has been found in the peripheral parts of gummata, especially at an

early stage of their formation; and it has been demonstrated in syphilitic disease of arteries, sometimes occurring in considerable numbers in the thickened patches in the aorta (Reuter, Schmorl; Wright *et al.*). That the spirochaete may persist in the body for a very long time after infection, has been abundantly shown by different observers; in one case, for example, its presence was demonstrated sixteen years after the primary lesion. Noguchi and Moore discovered the spirochaete in the brain in general paralysis of the insane in a proportion of cases. The organism was seen in all the layers of the cerebral cortex, with the exception of the outermost, and the cases in which it was found had run a relatively rapid course. It occurs in scattered clumps which may be very hard to find (Fig. 138).

CULTIVATION. Although Mühlens and Hoffmann had previously obtained pure cultures of an organism morphologically identical with the *Tr. pallidum*, Noguchi has worked most extensively on the subject and his results will be stated, although in certain respects they differ from those of the others

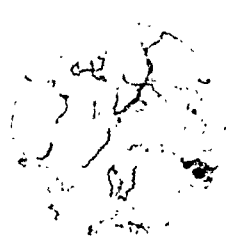


FIG. 138 Section of brain in general paralysis showing a clump of *Treponema pallidum*. Jahnke's method $\times 1,000$

the *Tr. pallidum* and all its characteristics. Pure cultures were grown in suitable (unheated) ascitic fluid, sufficient being placed in an 8 in. by $\frac{1}{2}$ inch test-tube to half fill (unheated) ascitic fluid, sufficient being placed in an 8 in. by $\frac{1}{2}$ inch test-tube to half fill

In the first instance cultures were made from syphilitic lesions in the rabbit, but later directly from the lesions of the human disease. The culture medium was a mixture of two parts of 2 per cent. agar and one part of ascitic or hydrocele fluid, to which a small portion of sterile rabbit's kidney or other organ was added, the medium being placed in deep tubes and covered with a thick layer of paraffin oil or vaseline through which the inoculation was made (The object of the only covering was to maintain anaerobiosis, but it may have prevented loss of gases from the medium). The contaminating bacteria which were present formed a thick growth along the line of inoculation, whilst the spirochaetes grew as a diffuse haze into the surrounding medium. By making subcultures from parts apparently free from bacterial growth the organism was obtained in pure condition. At first the organisms were small, but after several days they had the usual length of

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Accordingly, it is by no means generally admitted that hitherto obtained in cultures are genuine *Tr. pallidum*

Experimental Transmission of the Infection. Although experiments had previously been made from time to time by different workers, in some cases with reported successful results, it is to the observations of Metchnikoff and Roux that we owe most of our knowledge; they carried out a large series of observations, and showed that the disease can be transmitted to various species of monkey. Of these the anthropoid apes are most susceptible, the

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the disease has been transmitted by material from all the kinds of syphilitic lesions in which spirochaetes have been demonstrated, including tertiary lesions and the blood in secondary syphilis. Inoculation is usually made by scarification on the eyebrows or genitalia, but other parts of the skin are also susceptible in the higher apes. The subcutaneous and other methods of inoculation, with the exception of intratesticular and intravenous, give negative results. The primary lesion is in the form of one or more indurated papules, in every respect resembling the human lesion. Along with this there are marked enlargement and induration of the corresponding lymphatic glands. The primary lesion appears on an average about thirty days after inoculation, and secondary symptoms develop in rather more than half of the cases after a further period of somewhat longer duration. These are of the nature of squamous papules on the skin, mucous patches in the mouth, and sometimes palmar psoriasis. As a rule, the secondary manifestations are of a relatively mild degree, and in no instance has any tertiary lesion been observed, though this may be due to the animals not having lived long enough. By inoculation from the lesions the disease may be transferred to other animals. The infection may also be produced in *Macacus* and other lower monkeys, but these animals are less susceptible, and, moreover, the inoculation to be successful must be made at the special sites mentioned above, secondary manifestations do not appear. The severity of the affection amongst apes would in fact seem to be in proportion to the nearness of the relationship of the animal to the human subject. The blood of the infected animals comes to give a positive Wassermann reaction. This may be the case even where no primary or other lesion develops, as has been observed in *Macacus rhesus* monkeys inoculated either on the eyebrows or intratesticularly, with strains from the infection in rabbits or with fresh human syphilitic material (Schlossberger and Schmitz).

Hansell, and afterwards Bertarelli, were successful in inoculating the eye of the rabbit from syphilitic lesions. The material used is introduced in a finely divided state either into the tissue of the cornea or into the anterior chamber, and syphilitic keratitis or iritis, or both, may result, there being a period of incubation of at least two weeks. Levaditi and Yamanouchi studied the stages in detail, and found that the spirochaetes remain in the in-

not due to the organism passing through some cycle of development, but simply to its requiring certain conditions for multiplying which are not supplied for some time. The testis of this animal is also a convenient site of inoculation, a syphilitic orchitis being set up, and in the exudate spirochaetes occur in great numbers. By this method the disease has been passed through long series of animals. Intra-testicular inoculation leads to general invasion of the body, and, as Brown and Pearce have shown, metastatic lesions occur, the development of which is favoured by certain circumstances, such as excision of the primary lesion. Spontaneous healing tends to take place after the infection has passed through a series of relapses, but even when all signs of the disease have disappeared virulent spirochaetes are still present in the body, especially in the lymph glands. Thus a strain of *Tr pallidum* which is infective for the rabbit can be maintained indefinitely by inoculating from time to time fresh animals intra-testicularly with a suspension of

ground-up popliteal lymph glands from one in which the infection has become latent spontaneously.

The intra-testicular method has proved of great value in testing the infectivity of suspected material, and by this means it has been shown that spirochaetes from gummata are not attenuated in virulence. Infection has been transmitted to the rabbit by *inoculation with the brain tissue* of general paralytics; but as a rule only negative results have been obtained in such experiments. According to Levaditi and Marie there are 'dermotropic' and 'neurotropic' strains of *Tr. pallidum*, which differ in their pathogenic properties; these observations are suggestive as possibly throwing light on the variations in the effects in the human subject, but they have not been confirmed. The spirochaetes even in early human syphilitic products often show little virulence for the rabbit. Sometimes, where the primary inoculation has not led to a lesion, increase in virulence of the spirochaetes for the rabbit has been attained by inoculating a second animal some weeks later with material from the popliteal lymph gland of the first. After several such passages a lesion containing spirochaetes has developed in the inoculated testis. But only a few strains seem capable of propagation indefinitely in rabbits, e.g. those of Nichols, Truffi, and several others. As well as the infectivity, the character of the lesions produced tends to vary with the strain. Uhlenhuth and Mulzer produced generalised syphilitic lesions in young rabbits by intra-cardiac inoculation with syphilitic material. They found also that the organism can pass through the placenta of the rabbit and infect the foetus. Further, the spirochaetes are able to penetrate normal mucous membranes in the rabbit; but frequently no primary lesion develops, also the course of the resulting infection is mild (Brown and Pearce). According to Chesney and Kemp, a pre-existing lesion of the skin, such as an old granulating wound, affords in rabbits a very favourable site for the development of a primary chancre.

In the mouse *Tr. pallidum* produces no obvious effects; but if a small piece of rabbit's testis rich in spirochaetes be implanted under the skin of a mouse the organisms invade and proliferate in the tissues, which, including the brain, are found still to be capable of infecting rabbits many months later.

The presence of the organism in the blood about a week before the appearance of the primary sore, has been demonstrated in the case of a donor whose blood used for a transfusion produced the disease (McCluskie).

Biological properties of Tr. pallidum as demonstrated by experimental transmission. The experimental production of the disease has supplied us with some further facts regarding the nature of the organism. It has been shown repeatedly that the passage of fluid containing *Tr. pallidum* through bacterial filters deprives it completely of its infectivity. Hinde and Elford, by means of graded filters of collodion membrane, have found that while *Tr. pallidum* will pass through a filter which retains *B. prodigiosus*, nevertheless it is held back when the porosity is less than 0.4μ . In other words, no evidence was obtained of the existence of a special filterable phase significantly smaller than the diameter of the spirochaete. It is also readily destroyed by heat, a temperature of 51°C . being fatal. On the other hand, defibrinated blood containing *Tr. pallidum* has been found still to be infective after keeping for forty-eight hours at room-temperature; and rabbit plasma to which spirochaetes virulent for the rabbit were added, has remained infective after keeping at 5°C . for six days (Selbie). *Tr. pallidum* remains alive and virulent after freezing at -78°C . for at least one year, but freezing combined with desiccation kills it in about a day; freezing at -10° to -20°C . does not preserve vitality for two months (Turner, 1938).

Immunity Phenomena. It has long been accepted that a person suffering from syphilitic disease generally does not develop fresh lesions on reinoculation of the skin, and this was shown by experimental methods to hold in the artificially produced disease in the ape. Such immunity, in the absence of sterilising treatment with anti-syphilitic drugs, was found to be associated with persistence of the original infection. But when, as the result of anti-syphilitic treatment, the experimental infection in monkeys was sterilised, susceptibility to reinoculation soon developed. Similarly a considerable number of cases in the human subject have been observed where, after treatment with salvarsan, a second attack of the disease has been contracted, the inference being that the first attack had been completely cured. Work on rabbits, however, has indicated that this view may require modification. It has been shown that after intra-testicular inoculation treatment short of effecting complete sterilisation of the infection may restore susceptibility to reinoculation, or 'superinfection' as it is termed, provided that the treatment is begun when the infection is recent. But when the infection has lasted for a considerable time—at least forty-five to ninety days in the rabbit—then even if it is sterilised by treatment, reinoculation in the ordinary way does not lead to the development of a fresh primary lesion, nevertheless the spirochaetes may in a proportion of such animals invade the tissues, which consequently become infective again for fresh animals. The cornea, however, is exceptional, since it does not share in the resistant state to the same extent as the skin (Chesney *et al.*). Such immunity in the rabbit has been found to be more pronounced when the original infection and the reinoculation are made with the same strain than when different strains are used on the two occasions.

Spirochaeticidal properties have not been demonstrated in the serum of the immune animals by most observers; and the same is true of other antibodies. Accordingly it has been concluded that here there is a form of tissue immunity which in the early stages depends on the persistence of the infection, but which when treatment is begun late remains effective even although the infection has been sterilised. More recently, Turner (1939) has succeeded in obtaining evidence of antibodies by injecting intracutaneously in rabbits an incubated mixture of serum of a chronically infected animal along with a suitable small dose of virulent spirochaetes. The result was either that no lesions developed or else smaller lesions than after similar mixtures in which the serum was derived from a normal rabbit. Heating the immune serum at 56° C. diminished its protective action. Spirochaetes obtained directly from the tissues of infected rabbits are not agglutinated *in vitro* by syphilitic serum, but after treatment with a weak concentration of antiformin for a short time they become specifically agglutinable (Tami).

It has already been noted that the secondary stage of syphilis is characterised by abundant diffuse lesions containing numerous spirochaetes, which heal with the formation of little connective tissue. On the other hand, the tertiary lesions, which tend to be sparser, are of larger size but contain fewer spirochaetes, and on healing much more connective tissue is formed. These differences have been explained on the basis of allergy developing along with immunity as the duration of the infection increases.

With regard to the artificial production of immunity, very little of a satisfactory nature has so far been established. It has been found that the organism after passage through a *Macacus* monkey produces a less severe disease in the chimpanzee than when derived directly from the human subject, inasmuch as secondary lesions do not follow; the organism would thus appear to have undergone a certain amount of attenuation in the tissues of

that monkey. But accidental infection of the human subject has occurred with spirochaetes which had been repeatedly passed through rabbits during a period of over a year and a half.

Luetin. Noguchi prepared an extract from pure cultures of the *Tr pallidum*, which he called *luetin*, and he found that this gave a characteristic cutaneous reaction in syphilitics, which is analogous to the tuberculin reaction in tuberculosis. Although the reaction may be of value for detecting latent syphilitic infection, it appears that it is not specific in the etiological sense. In positively reacting subjects other substances, e.g. agar or killed cultures of bacteria, may produce a similar reaction. Again, non-syphilitic individuals come to react positively as a result of the administration of iodides (Sherrick).

Serum Diagnosis—Wassermann Reaction and Flocculation Reaction. Wassermann, Neisser and Bruck, proceeding in accordance with the facts established with regard to the fixation of complement, tested whether a similar phenomenon might not be obtained in the case of syphilis. They mixed together a watery extract of congenital syphilitic liver rich in spirochaetes (antigen), and serum from a syphilitic case (supposed to contain antibodies), and found that a relatively large amount of complement was fixed. On the other hand, when the serum from a non-syphilitic case was substituted for the syphilitic serum, little or no fixation of complement occurred. The result was thus in accordance with expectations on theoretical grounds. Marie and Levaditi, however, found that an extract of a normal infant's liver along with syphilitic serum fixed complement, i.e. acted as 'antigen'. Subsequent observations (Porges and Meier *et al.*) showed that alcoholic extracts of normal as well as syphilitic tissues are also very efficient, and that the property resides mainly in the fraction of the extract which is soluble in alcohol and ether but insoluble in acetone (lecithin). The addition of cholesterol to lecithin enhances greatly its activity (Browning, Cruickshank and Mackenzie). Although abundant observations have established the validity of the test as a means of diagnosis, the reaction which led to its discovery is no longer sufficient to explain it. The occurrence of precipitation or flocculation when syphilitic serum is mixed with lipoids or other colloids was also noted; and the conditions have been defined in which this reaction constitutes a reliable diagnostic test.

The method of applying the Wassermann test is described in the Appendix; here we have to consider the results of its application. It will not be an over-estimate to say that a positive result may be obtained in at least 90 per cent. of cases where there is evidence of active general infection. The reaction usually appears first on the tenth to thirtieth day after development of the sore, and then gradually becomes more marked, during the period of secondary manifestations it is practically always present; in the tertiary stage with active manifestations a positive result is a little less frequent. In cases of congenital syphilis with active lesions the proportion of positive results is nearly as great as in the secondary stage of the acquired disease.

It may only have become latent, and on its reappearance the reaction may reappear, in fact, its presence would appear to be definitely related to the activity of the syphilitic infection. Accordingly, the reaction is likely to be positive when there are active generalised lesions. As regards neurosyphilis, a positive reaction is practically always present in general paralysis and in the large majority of cases of tabes, and is as frequently given by the cerebro-spinal fluid as by the blood serum in these diseases. In certain cases of neurosyphilis the cerebro-spinal fluid may react

positively when the blood serum is negative. As to the occurrence of positive reactions in other diseases, this has been recorded in yaws, pinta, and bejel, also in leprosy, sleeping-sickness, and occasionally in malaria during the febrile periods. Apart from these diseases 'false positive' reactions are seldom met with, although vaccination and various febrile conditions, especially respiratory infections and infective mononucleosis and also the injection of foreign serum, tend to cause temporarily positive reactions, usually weak. (For further information on fallacies of the serological reactions for syphilis the critical reviews of Harrison and Osmond should be consulted.) At present little can be said in explanation of the Wassermann reaction. It seems to depend on the interaction of lipoidal substances in the extract with proteins in the serum, which are apparently contained in the globulin fraction. It is now accepted that it does not depend on the presence of an immune-body which in association with the causal agent (the spirochæte) fixes complement. However, there is considerable evidence that the reacting power of the serum is due to an antibody for certain lipoids (see Dunlop and Sugden, Mackie *et al.*); and it may be that the same antigenic constituents are present in the spirochætes and the tissues (*vide infra*). But in that case we do not know why an antibody should be formed to components of the patients' own tissues.

The various forms of flocculation reactions (Sachs and Georgi, Mehncke, 'Sigma' of Dreyer and Ward, Kahn, 'Citochol'; etc.) give results which are comparable with those of the Wassermann test—the methods of such tests are described in the Appendix. A small proportion of sera from cases with a history of syphilis react positively in the flocculation test but negatively in the Wassermann reaction, while occasionally the converse occurs, discrepancies occur especially in cases undergoing anti-syphilitic treatment. Thus, neither the Wassermann test nor the flocculation test alone will detect all syphilitic sera, but when a series of sera is examined both yield practically the same number of positive results. The most complete information is obtained when the two tests are used in conjunction, the one serving as a confirmation of the other, especially when a weakly positive result occurs. No single one of these tests can be relied on to exclude syphilis on the basis of a negative result. Although the technique of the various flocculation methods is relatively simple, accurate preliminary standardising of the 'antigen' is essential and experience in reading the results is required.

Bacteriological Diagnosis. Demonstration of the characteristic spirochætes by microscopic examination is necessary where an ulcerated lesion of the skin or a mucous membrane is suspected to be a primary sore. It may also be used in the case of an enlarged lymphatic gland, e.g. that draining the site of a suspected chancre which has healed, or for secondary lesions. In the case of tissues, e.g. in congenital syphilis, an emulsion in saline may be examined similarly. Wherever there is contamination with surface secretions other treponemata may be present, some of which are almost indistinguishable from *Tr. pallidum* or differ chiefly in their greater size (*vide infra*). Accordingly, it is essential always to work at the same magnification. Further it may be said that in the microscopic diagnosis of the organism of syphilis, just as in the case of the tubercle bacillus, an all-important point is the source of the organism. Excepting the case of yaws, which does not occur in this country, an organism with the characters described above can be identified with certainty as the *Tr. pallidum* provided that the superficial organisms are removed and the exudate is obtained from the substance of the tissue lesion. The best method of examination is by dark-ground illumination since in

stained films the apparent thickness of the spirochæte depends on the intensity of staining and this may introduce a difficulty in distinguishing it from other similar organisms.

In the examination of exudate from an ulcerated chancre or other lesion it is advisable first to get rid of the surface organisms. The surface should be cleansed with saline and dried. A piece of cotton wool soaked in absolute alcohol or spirit is then applied for about a minute, the alcohol is then washed off with saline, and the surface is again dried. After a short time there is usually a free flow of watery serum, which is practically free

scarify the margin of the sore and examine the lymph which exudes, the flow of which may be aided by squeezing, or a small incision may be made with a very sharp knife, and then after bleeding has completely stopped the small drop of serum is taken which gathers at the site. In all cases admixture of blood is to be avoided, as it interferes with the examination by the dark-ground method. It is important that neither local antiseptic applications nor any form of antisypilitic treatment should be given before the examination is made. In the case of a lymphatic gland or non-ulcerated lesion, it is best to puncture with a hypodermic needle, the point of which should be moved about in the tissue. After it is withdrawn a little saline may be placed in the syringe and pressed through the needle, the first small drop which passes, and which washes out the contents, being taken for examination; here also dark-ground illumination gives the best results. An emulsion of tissue in saline may also be examined by dark-ground illumination. Tissues are fixed in 5 per cent formalin and treated by Levaditi's or Jahnke's methods.

Dried films may be made and stained by various methods, Becker's is recommended.

The inferences to be drawn from serological examinations have been discussed above.

TREPONEMA PERTENUE

Frambœsia or yaws is a disease of the tropics, occurring in the west coast of Africa, Ceylon, the West Indies, and other parts. It is characterised by

a distinctive cutaneous eruption, and it is markedly contagious. Its resemblance in many respects to syphilis has been noted, and the relation of the two diseases has been the subject of much controversy. It is accordingly a matter of great interest that an organism morphologically practically identical with the *Tr. pallidum* was discovered by Castellani in the lesions of frambœsia (Fig. 139). He gave to it the name *Spirochata pertenue* or *pallidula* (*Treponema pertenue*, Castellani). In the skin lesions it has been shown by Levaditi's method to be present in considerable numbers, especially in the epidermis and also amongst the leuco-

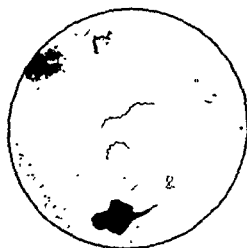


FIG. 139. *Treponema pertenue*. Film preparation from skin lesion in yaws, stained by Becker's method $\times 1,000$

seen in the case of syphilis. When ulceration has occurred chætes of other types may be present as contaminants. Infection is transmitted by contact, but not venereally, congenital infection does not occur. Insects may also be the vectors sometimes, since the spirochæte has been

found to survive for some hours in the anterior part of the alimentary tract of certain flies. Patients suffering from frambæsia generally give a positive Wassermann reaction; the lesions are also very amenable to treatment with salvarsan (Alston and others)

Castellani found that the disease could be transferred to monkeys (*Semnopithecus* and *Macacus* being used for this purpose), and that the organism could be demonstrated in the unbroken skin lesions. The lesions are as a rule confined to the site of inoculation, but the infection is general, as is shown by the presence of spirochaetes in the lymphatic glands and the spleen, although they do not survive in the lymphatic fluid after the skin has healed from *Tr. pertenue*.

Tr. pertenue in the lesions, and the inoculation of apes have been confirmed by other workers, and the etiological relationship of the organism to the disease may now be regarded as established. Nichols has shown that a frambæsia lesion can be produced in the testicle of the rabbit of similar character to the syphilitic lesion, though the period of incubation is shorter. He found that the best means of distinguishing the two infections was afforded by inoculating the skin of the monkey. In the case of syphilis the resulting lesion is flat, dry, and very scaly, in the case of frambæsia it is elevated, slightly scaly, and very oedematous, and also the period of incubation is shorter in the case of frambæsia. Pearce and Brown observed on intra-testicular inoculation with *Tr. pertenue* in the rabbit a granular or finely nodular peri-orchitis—a type of lesion quite different from that observed in the case of *Tr. pallidum*. Similarly, Turner and Chesney working with a number of strains of *Tr. pertenue* isolated from cases in Haiti, found that in rabbits the characteristic initial testicular lesion consisted of scattered miliary granules in the substance of the organ, the tunica, or the epididymis. The great enlargement or marked induration, so common after inoculation with *Tr. pallidum*, did not occur. On intracutaneous inoculation, again, a superficial lesion without induration resulted, in contrast to the raised indurated button-like chancre due to *Tr. pallidum*. It is noteworthy that a strain of the syphilitic spirochaete isolated in Haiti at the same time as the frambæsia strains, showed the behaviour in rabbits which is characteristic of other strains of *Tr. pallidum*, this, however, does not affect the fact that different strains of *Tr. pallidum* may vary as to the character of the lesions which they produce. Further, in frambæsia in rabbits there is not the tendency to development of generalised lesions which is characteristic of syphilis. According to Ferris and Turner, however, there is no reliable means of distinguishing the lesions of yaws and syphilis histologically.

The immunity reactions in monkeys and rabbits infected with syphilis and frambæsia (yaws) have been experimentally studied with contradictory results. According to the thorough investigations of Schöbl and Miyao monkeys which had recovered from yaws and were immune to reinoculation with *Tr. pertenue* developed only very slight lesions on inoculation with a strain of *Tr. pallidum* which was virulent for the rabbit and for normal monkeys. Further, the syphilis spirochaetes were not able to persist in the tissues of monkeys immune to frambæsia as was shown by the non-infectivity of the lymph glands of the latter for rabbits. Schöbl has also recorded observations regarding the influence exerted by inoculation with *Tr. pallidum* on a pre-existing latent infection with *Tr. pertenue* in monkeys. Animals which had been injected with killed *Tr. pertenue* developed no local lesion on repeated inoculation of the skin with these organisms in the living state. Later, after an inoculation with syphilis spirochaetes no lesion developed at

subcultures. Growth does not occur in the absence of oxygen. It seems that after a number of passages cultures tend to die out.

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microscope, these being actively motile with many dividing forms (The addition of 5 to 10 drops of 10 per cent glucose solution to a tube of medium seemed to improve it) A paraffin seal did not improve cultures Subcultures were readily got by adding 3 or 4 drops of a culture containing numerous spirochætes and at the same time 3 or 4 drops of citrated or freshly drawn rabbit's blood to a tube of medium Twelve successive subcultures at intervals of two to four days led to no loss of vitality of the spirochætes

Inoculation of the chorio-allantoic membrane of developing hens' eggs with the spirochæte leads to massive infection of the blood of the embryo chick. A strain of *Bor duttoni* retained its infectivity for mice after seventeen serial passages in eggs (Oag).

Relations to the Disease. Before the rise of temperature there is a period of incubation varying from two to twelve days or longer after inoculation. The organisms begin to appear in the blood shortly before the onset of the pyrexia, and during the rise of temperature rapidly increase in number. They are sometimes very abundant during the fever, a large number being often present in every field under the high power of the microscope. In some cases, however, they may be less numerous and even difficult to find by microscopic examination; this is especially true of African relapsing fever. They begin to disappear shortly before the crisis; during the afebrile interval they commonly cannot be found in the circulating blood on microscopic examination, but the blood is, as a rule, still infective. A similar relation between the presence of the organisms in the blood and the fever is found in the relapses; but here the organisms tend to be less numerous than in the first attack. The spirochæte may be present in the cerebro-spinal fluid with or without evidence of the meninges being affected. Munch in 1876 produced the disease in the human subject by injecting blood containing the spirochætes, and this has been repeatedly confirmed. Recently inoculation has been practised as a therapeutic measure in general paralytics (cf p. 482). Accidental infection has occurred through the apparently intact skin or the conjunctiva. Carter in 1879 was the first to show that the disease could be readily produced in monkeys, and these experiments were confirmed by Koch. The blood taken from patients and containing the spirochætes was injected subcutaneously into the monkeys. In the disease thus produced there is an incubation period which usually lasts about three days. At the end of that time the organisms rapidly appear in the blood, and shortly afterwards the temperature quickly rises. The period of pyrexia usually lasts for two or three days, and is followed by a marked crisis. As a rule there is no relapse, but occasionally one of short duration occurs. Cunningham, working with strains from Indian relapsing fever, found that in monkeys a relapse frequently occurred. He points out that in animals relapses are often transient and may easily be missed. White mice (and often rats) are also susceptible to infection, spirochætes becoming numerous in the peripheral blood and then disappearing after several days, often without illness resulting. After repeated passages in these animals relapses may occur regularly; but when the infection has

been recently derived from man there is often only a single attack with disappearance of the organisms by crisis. In mice the infection tends to persist in a latent state in the brain. Various other rodents are susceptible to inoculation. Cunningham in India has used squirrels; these, after inoculation, develop a primary infection which is frequently followed by a single relapse, as in the human disease, a second relapse being rare. When relapsing fever is contracted during pregnancy the fœtus may become infected.

Immunity. Metchnikoff found that during the fever the spirochætes were practically never taken up by the leucocytes in the circulating blood, but that at the time of the crisis, on disappearing from the blood, they accumulated in the spleen and were ingested in large numbers by the macrophages and polymorphonuclear leucocytes. Within these they rapidly underwent degeneration and disappeared. It is to be noted in this connection that enlargement of the spleen is a very marked feature in relapsing fever.

chæte, it was necessary to inoculate animals whose spleens had previously been removed. This property of the spleen has been related to its large content in cells belonging to the reticulo-endothelial system.

As in the case of so many other infections, however, an all-important factor in the destruction of the organisms is the development of antibodies in the blood. Lamb showed in the case of the monkey (*Macacus radiatus*) that the immunity following an attack of the disease was due to the presence of spirochætidal bodies in the serum. He found that *in vitro* the serum of an immune animal brought the movements of the spirochætes to an end, clumped them, and caused their disintegration, and further, that in one case when the spirochætes and the immune serum were injected into a fresh monkey, no disease developed. Novy and Knapp produced a 'hyper-immunity' in rats by repeated injections of blood containing the spirochætes, and found that the serum of such animals had a marked curative effect, and could cut short the disease in rats, mice, and monkeys. It has also been de-

fixation reaction has been got with serum from cases of relapsing fever (Stein). This antigen showed a broad species specificity and not a restricted strain specificity in reacting with the serum antibodies. Brüssin showed that with these organisms an immunity phenomenon can be demonstrated similar to that with trypanosomes: when antibody-containing blood is added to the homologous spirochætes the latter become coated with blood platelets, or if antiserum and a culture of bacteria, e.g. *B. coli*, are mixed with the spirochætes the latter become coated with the bacteria—the adhesion phenomenon. It is best seen by dark-ground examination.

The relapsing course which is characteristic of the human disease has been explained on the basis of a transient immunity developing during the first period of pyrexia, some of the spirochætes, however, in internal organs or in tissues escaping destruction by the serum or by phagocytes. These organisms are then able to multiply and reappear in the blood, possibly in part as a result of disappearance of the immunity, but chiefly because a strain has developed which is resistant to the protective antibodies resulting from the original attack (Levaditi and Roché). In the case of Indian relapsing fever, in which there is usually only one relapse, Cunningham *et al.* have shown that the strain of spirochæte present in the relapse is serologically distinct (as

judged by agglutination reactions) from that observed in the original attack, and the same holds good for experimental infections. Further, experimental infection with one of these two serological types is followed by a relapse due to the other, the types present in the original attack and in the relapse alternating regularly. But where repeated relapses occur additional antigenic types appear or there may be reversion to an earlier type. Also, antigenic variants may develop in the course of a single attack. These observations indicate that the antigenic character of the organism undergoes alteration by the influence of the defences of the body, and offers an explanation of the relapsing nature of the infection. The immunity reactions above described depend on a very delicate adjustment between the antibody and the parasite. Thus passage through a different host, as well as the influence of the latter at different stages of the infection, tend to modify the antigenic properties of the spirochætes. Accordingly, these reactions have little value for the identification of different species. The immunity following an attack in man disappears within several years.

Chicks almost immediately after hatching are, like adult fowls, quite resistant to infection with *Bor. duttoni*, whereas the embryo chick is highly susceptible, as mentioned above. The difference does not appear to be explicable on the basis of properties of the whole blood or serum demonstrable *in vitro*, since both are spirochæticidal when derived from the susceptible chick embryo (and similarly from the mouse), as well as from the refractory chick (Oag).

Varieties of Relapsing Fever Spirochætes. As already stated, relapsing fever has been studied in different parts of the world and differences have been made out with regard to clinical features, pathogenic effects in man and on experimental inoculation, arthropod vectors and immunity reactions. It has been emphasised by Brumpt that points of special importance in distinguishing the different forms which is capable of acting

mental animals to infection, especially the guinea-pig. The latter is infected with louse-borne spirochætes, but may be infected with some of those which are tick-borne. Apart from tick-borne fever, European, Asiatic, and North African types have been distinguished, all of which are transmitted by lice (*vide infra*). Relapsing fever in Asia is apparently a much more severe disease than in Europe; F. P. Mackie gave the mortality in Bombay at the high figure of 38 per cent.; but differences in this respect, as well as in pathogenic effects, may depend on variations in virulence of the spirochæte or on

the nature of the animal attacked. The effect of under-nutrition, well recognised. African tick-fever is transmitted by the bites of arthropods suggested that European and Asiatic relapsing fever is transmitted in this way. At first the bed-bug was believed to be the vector of transmission, but attempts to transmit the disease by means of the bites of bugs were generally unsuccessful; F. P. Mackie produced the infection in only one out of six monkeys used for this purpose, though large numbers of bugs which had bitten relapsing fever patients were used. On investigating an epidemic of the disease, however, he obtained a considerable amount of evidence on epidemiological grounds that the disease was carried by the body louse (*Pediculus humanus*, var. *corporis*). This has been established by the work of Nicolle, Blaizot and Conseil, and by the experiments of Manteufel, who was able to transmit infection from rat to rat in nearly 60 per cent. of the experiments, whereas he obtained only negative results by means of bugs.

The mode of inoculation of the organism from infected lice is by its introduction into abrasions produced by scratching—the crushed insect providing the inoculum. Infection may also occur through intact mucous membranes. The spirochætes can be observed in the stomach of the louse for a day after infective blood has been ingested, after about six days they can be demonstrated in the body cavity, and then spread throughout the insect's body and legs. It has been supposed that the organisms may be infective when in a non-spirochætal stage (Sergeant and Foley). Clear evidence is lacking that infected lice transmit the spirochæte to their ova.

The spirochæte of Indian relapsing fever has been called *Bor carteri*, while the designation *Bor obermeieri* may be retained for the European variety. Sergeant and Foley have described a type of relapsing fever in Algiers and have given the name *Bor berbera* to the organism concerned. A form of relapsing fever similar to the European and Asiatic types and also transmitted by lice, occurs in West Africa. It has been concluded by some workers that there are probably several distinct species of these spirochætes, as animals immunised against one are still susceptible to infection with others and vice versa. But the limited value of such observations has been indicated above. In general, there is no strong evidence that the louse transmits more than one species of relapsing fever spirochæte.

Tick Transmission. Spirochætes had been seen in the blood of patients with tick-fever in Uganda by Greig and Nabarro in 1903, and Ross and Milne in 1901 recorded a series of observations which led them to the conclusion that tick-fever was due to a spirochæte. It is, however, chiefly owing to the work of Dutton and Todd in the Congo Free State, on the one hand, and of Koch in German East Africa, on the other, that our knowledge of the etiology of the disease has been obtained. Thus the disease long known by the name of Tick-Fever as prevalent in Tropical Africa has been shown to be caused by a spirochæte—*Bor duttoni* (*Bor crociduræ*) (Fig. 144).

The following are the chief facts regarding this disease. Clinically, the fever closely resembles relapsing fever, but the periods of fever are somewhat shorter, rarely lasting for more than two or three days, and the relapses are more numerous. The organisms in the blood are considerably fewer than in the case of European relapsing fever, and sometimes they can only be demonstrated after prolonged search or by inoculation of a susceptible animal with the patient's blood. Dutton and Todd showed that it was possible to transmit the disease to certain monkeys (*Cercopithecus*) by means of ticks which had been allowed to bite patients suffering from the disease, the symptoms in these animals appearing about five days after inoculation. The disease thus produced is characterised by several relapses, and often leads to a fatal result. Ticks can be infected at any stage of development as a result of biting infected persons. In one case the disease was produced by means of young ticks hatched from the eggs of ticks which had been allowed to suck the blood of infected patients, hence it was concluded that the spirochætes were not simply carried mechanically by the ticks, but probably underwent some cycle of development in the tissues of the latter. Leishman also showed that the



FIG. 144. Film of human blood containing spirochæte of tick-fever, *Bor duttoni* $\times 1,000$. (From a preparation of the late Lt.-Gen. Sir William Leishman, A.M.S.)

ticks of the second generation may be infectious. The species of tick concerned are chiefly *Ornithodoros moubata*, also *O. savignyi* and sometimes *O. erraticus*. Koch found that after the ticks had been allowed to suck blood containing the organisms, these could be found for a day or two in the alimentary tract of the tick; then of the gut. At this stage they the coelomic fluid. Thence the salivary glands and the ovaries. At the latter site they were detected by Koch in large numbers, sometimes forming felted masses. He also traced the presence of the spirochætes in the eggs laid by the infected ticks, and in the young embryos hatched from them. On the other hand, Leishman failed to find evidence of spirochætes either in the tissues of ticks later than ten days after ingestion of infected blood, or in the ova laid by the ticks, or in the young ticks when hatched, though these were proved by experiment to be infective. After ingestion of the blood by the ticks, he found that morphological changes occur in the spirochætes, resulting in the formation of minute 'chromatin granules' which are extruded and traverse the walls of the intestine and are taken up by the cells of the Malpighian tubules; they also penetrate the ovaries and may be found in large numbers within the ova. Similar granules are to be seen in the Malpighian tubules of the embryo ticks, where they are also found in the subsequent stages of their life. He recorded that infection of animals might be produced by inoculation with crushed material containing the granules but no spirochætes. Accordingly he considered that the granules in question represent a phase in the life-history of the parasite, and that infection occurs by inoculation of the skin with the chromatin granules voided in the coxal secretion and not by unaltered spirochætes from the salivary glands. A similar view was taken by Hindle, who found that when infected ticks in which the spirochætes had disappeared, were warmed to a temperature of 35° C, the spirochætes reappeared in the organs and coelomic fluid. These 'metacyclic' forms differ from those in the blood in being thinner, shorter, more irregular, and less deeply staining. Others, however, hold that there is no such cycle (see Bone). The coxal secretion, saliva, and faeces are the sources of infection.

According to Koch's observations along the main caravan routes in German East Africa, 11 per cent of the ticks contained spirochætes, and in some localities almost half were infected. In places removed from the main lines of commerce he still found them, though in smaller number. It has also been demonstrated that in some places the ticks are infected with the spirochætes although the inhabitants do not suffer from tick-fever a circumstance which is possibly due to their having acquired immunity against the disease. It is important in connection with the transmission of the disease that infected ticks have conveyed the spirochæte to monkeys on which they fed after

it is able to produce infection in the guinea-pig, *obermeieri*. Experiments have indicated also, that there is a specific relation between each parasite and its invertebrate host, the louse-borne infection is not transmissible by ticks. Differences brought out by immunity reactions both *in vivo* and *in vitro* have been adduced as evidence by Breinl and others that these spirochætes belong to distinct species. As has been stated already,

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however, the variability which a given strain of spirochæte may exhibit renders the method of doubtful value

Tick-borne relapsing fever also occurs in Spain, the United States, and Central and South America, Arabia, and Persia. The Spanish form, due to *Bor. hispanica*, is a mild disease, it is transmitted by *O. erraticus*. In the United States and Central America sporadic infections occur, it is of importance that in this region small rodents naturally harbour the spirochæte and so may maintain the infection in the ticks which infest them. In the U.S.A., California and Texas are especially affected, the vectors being *O. turicata*, *O. hermsi*, and *O. parkeri*. *Bor. persica* (of the Persian disease) appears to be transmitted directly by the bite of *O. papillipes*, since it does not excrete coval fluid or faeces while biting, and this is true of certain other ticks also. It remains undecided how far the spirochætes found in different localities and transmitted by particular ticks are distinct species

Bacteriological Diagnosis. The spirochæte may be demonstrated readily in the blood in the louse-borne infection during the febrile period and also in a considerable proportion of cases between relapses, dark-ground examination is best, but stained films serve. In tick-borne fever the organisms are scantier, wet films should be thin, as an excess of red corpuscles obscures the spirochætes. Such a film should be searched for at least ten minutes. Scanty organisms may be demonstrated also in thick films, dried and dehaemoglobinised before staining, as for malarial parasites. For staining Leishman's fluid or other Romanowsky stains or simple solutions of basic dyes, e.g. crystal violet, are suitable. Field's thick film method, as used for malaria, is also valuable. Injection of 1 to 2 c.c. of fresh blood containing scanty parasites or of blood clot broken down in saline, into a mouse intraperitoneally may produce infection (a contaminated specimen should be injected subcutaneously). The spirochætes tend to be more virulent in blood taken during the early phase of the attack. A drop of blood from the tail of the mouse should be examined daily for several weeks. The complement-fixation reaction with the patient's serum and a suitable antigen consisting of spirochætes, has been described above. The serum of relapsing fever patients is stated to give a positive Wassermann reaction in a proportion of cases, but it is doubtful whether positive reactions are obtained in the Weil-Felix test. The spirochæte may be demonstrated in ticks by making smears of the contents of the stomach and various tissues. Lice are kept in a test-tube for twenty-four hours after removal from the patient, then placed with fine forceps in a small drop of distilled water on a microscope slide and the abdomen transfixed by a needle lateral to the gut, so that the hæmoele fluid escapes into the water. This is examined by dark-ground illumination or dried films are made and stained.

BORRELIA VINCENTI

An ulcerative form of throat and mouth infection characterised by soft greyish membrane and foul smell, with much œdema around, was recognised by Plaut and Vincent to be associated with large numbers of spirochætes along with fusiform bacilli (p. 468). The spirochætes, which are Gram negative and stain faintly with watery solutions of basic dyes, resemble *Bor. refringens* (p. 490). The condition is generally known as Vincent's angina. In ulcerative gingivitis and stomatitis the same two types of organisms are numerous, and the occurrence of small epidemics of this affection is suggestive of an infective origin. It must be noted, however, that pre-existing pathological conditions in the mouth such as excessive deposits of tartar and

carious teeth predispose to the lesions. Similar spirochætes are found in certain tropical ulcers, noma (*cancrum oris*), phagedænic ulcerations of the genitalia and in putrid types of bronchial and lung infections; they have been named variously *Bor. vincenti*, *Bor. phagedenis*, *Bor. balanitidis*, etc. It is not clear whether one or more species of spirochætes are concerned; Tunnicliff's view that they are merely a different form of the fusiform bacilli has not been accepted generally. The fact that similar spirochætes occur in the healthy mouth raises the question whether this organism is really a pathogenic agent or whether its presence in ulcers, etc., merely represents an overgrowth of commensals, e.g. in true diphtheria they may be found in large numbers. The response to treatment with arsenicals (either general or local) and penicillin has been considered as evidence of the spirochætes acting as pathogens. In a section of the lesion when stained with methylene blue or thionin, e.g. in *cancrum oris*, at the advancing margin the spirochætes are found deepest in the tissue, then spirochætes and fusiform bacilli, although necrosis takes place only after the appearance of the latter. At present it seems sufficient to class all those conditions in which the spirochætes and fusiform bacilli are found as 'Vincent's disease'.

PSEUDOSPIROCHÆTES

In blood, especially after it has been heated *in vitro* at 37° C. and upwards, filamentous structures arise from the red cells, etc., which on examination of wet films by dark-ground illumination show a waving motion; they may be beaded. Such structures are liable to be mistaken for spirochætes. Their 'worm-like' character and the absence of spinning

tions of these, may present appearances indistinguishable from spirochætes.

CHAPTER XXV

LEPTOSPIRA ICTEROHÆMORRHAGIÆ, ETC. SPIRILLUM MINUS

LEPTOSPIRA ICTEROHÆMORRHAGIÆ

A FORM of acute infective disease characterised by jaundice, enlarged spleen, and nephritis had been recognised by Weil and was known as Weil's disease or infective jaundice. Inada and other Japanese workers in 1915 proved that it was due to a spirochæte, to which they gave the name *Spirochæta icterohæmorrhagiæ*. This spirochæte is characterised by its exceedingly numerous and fine, closely wound coils, and in 1917 Noguchi applied to it the generic name of *Leptospira* on account of its morphology. The infection has been shown to have a wide distribution throughout the world, it is practically always produced in man by contact with infected animals or their urine. Other leptospiræ pathogenic for man have been discovered and also non-pathogenic leptospiræ occur in water. These organisms are differentiated chiefly by their serological reactions. The clinical features of the diseases which they cause are not a reliable means of distinguishing them, since mild infections produced by *L. icterohæmorrhagiæ* take the form of an indefinite febrile illness without jaundice and with low mortality, resembling 'influenza' or diseases commonly caused by other leptospiræ, while severe infections are highly fatal and have been confused clinically with yellow fever. The disease caused by *L. icterohæmorrhagiæ* is characterised by the sudden onset of symptoms, general malaise, severe headache, and muscular pains, with high fever lasting about ten days, a tendency to hæmorrhage from mucous surfaces, conjunctival congestion, and, in severe cases, jaundice which becomes increasingly marked from about the fourth day of the illness. Although jaundice was originally regarded as a cardinal feature, it is now known that it is present in only about half the cases, these being the most serious. There is a marked polymorphonuclear leucocytosis. The excretion of nitrogen by the kidney is impaired. Meningitis has been recorded in this infection. In convalescence relapses may develop. The occurrence of the disease in small epidemics had been previously noted. In Japan it was found to occur among workers in the same part of wet mines. During the war of 1914-18 troops in France were affected and the results of the Japanese workers were confirmed by bacteriologists in both the British and French armies. The mortality was much lower than that met with in Japan. On the Continent the disease has in recent times been shown to have a wide endemic distribution. It was first described in Scotland by Gulland and Buchanan among coal miners working in a wet mine, and Davidson and others observed it among fish workers, farm workers are also frequent among those attacked (Gardner). It has occurred in sewer workers in London (Fairley). In Holland there have been frequent cases, especially following immersion in canal water. The incidence of the disease is accounted for by the usual source of the infection, namely, the urine of rats which are carriers of the spirochæte.

MICROSCOPIC CHARACTERS. The organism in the blood and tissues measures 6 to 9 μ in length (but both shorter and longer forms occur) and about 0.25 μ in thickness—that is, it is a slender organism of about the thickness of the *Tr. pallidum*. In cultures it may grow into much longer forms. It is somewhat thicker in the middle and tapers towards the ends, which

may be pointed, but there are no terminal flagella (Figs. 145, 146). The morphology is best demonstrated by dark-ground illumination and is characterised by numerous fine 'elementary' spirals and recurved or 'hooked' ends,



FIG. 145. *Leptospira icterohæmorrhagiæ* in a renal tubule of a wild rat: section stained by Levaditi's method. Note the typical hooked ends of one of the spiracles. $\times 1,000$ (approx.)

FIG. 146. *Leptospira icterohæmorrhagiæ*, from a culture in Noguchi's medium. Stained by Kirkpatrick's method $\times 1,000$

while occasionally secondary spiral. In fixed and stained material the organism is motile and strabale.



FIG. 147. *L. icterohæmorrhagiæ* as seen in a culture by dark-ground illumination, showing elementary spirals. (From a drawing by Dr Geo Buchanan.)

The limits of growth are wide, namely, 13° to 37° C., the optimum temperature being 25° to 30° C. The following are suitable media.

Noguchi's medium. (a) A mixture of 0.9 per cent. NaCl solution, rabbit's blood, 1 part (b) Tinction of one part of neutral or acid and the ingredients mixed with uniform mixture. Both media are suitable.

The organism is being rotatory and undulatory. It can be studied by all the microscopic methods already described in the case of the *Tr. pallidum* (p. 479).

VIABILITY A suspension of the organisms is killed by exposure to 50° to 55° C. for half an hour. In the liver of an infected guinea-pig they may remain alive and virulent when kept in the ice-chest for over three weeks. They are highly sensitive to the acidity of gastric juice, to bile and antiseptics.

CULTIVATION The organism was first successfully cultivated in Noguchi's medium for *Tr. pallidum*, in which the initial growth survived for three to six weeks. Later it was grown in various semi-solid and fluid media.

In order to get a layer of sterile liquid paraffin, and to Buchanan medium (b) gives most

Cultures for agglutination tests are obtained as follows, according to Gardner. Rabbit's serum, after heating for one hour at 56° C., is passed through a Seitz filter, then diluted with glass-distilled water (traces of copper must be absent from the medium) to a 12 per cent concentration and distributed in 5 c.c. amounts in tubes. A heavy inoculum of an actively motile culture is required (0.5 to 1 c.c. per 5 c.c. of medium), the subculture should yield a rich growth in four or five days at 30° C., which is suitable for agglutination. Cultures live for about a month at room temperature, but for maintenance in a suitable state should be subcultured weekly. According to Stuart slight lysis of the rabbit's blood from the addition of 1 c.c. sterile water to 20 c.c. blood improves the medium. The serum of certain rabbits inhibits growth of the leptospira.

Stuart's medium serves both for isolation and maintenance of the leptospira and for cultures for agglutination tests. Distilled water is used throughout. Of M/10 solutions of the following reagents, the amounts in c.c. shown are measured with aseptic precautions in the order indicated and mixed—*asparagine* (J. rot.) 2, NH_4Cl 10, MgCl_2 4, NaCl 60, then pure glycerol 1, 0.02 per cent aqueous solution of phenol red 10 and water 91. The mixture and also Sörensen's buffer solution pH 7.6 ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 11.876 grms per litre, 17.6 c.c. added to KH_2PO_4 9.078 grms per litre, 2.4 c.c.) are steamed separately for thirty minutes, then 16 c.c. of the buffer solution are added to the mixture and the whole is steamed for one hour further (this medium keeps for about one month but much longer if stored in completely full bottles. Two or 3 c.c. of the medium are pipetted aseptically into sterile screw-cap bottles (which must be free from traces of soap and should have been soaked in the above buffer solution for twenty-four hours and then rinsed with water and sterilised). 5 to 10 per cent of rabbit serum (previously filtered and checked as to the pH being unaltered) is added and the bottles are heated in a water bath at 60° C. for one hour (if tubes plugged with cotton wool are used as containers the plugs must not be flamed). Inoculation and incubation are carried out as described above under Gardner's method.

Relation to the Disease. The incubation period, as shown by infections due to accidental immersion, is about ten days. The organism occurs both in the blood and in the organs. From the former it can be recovered by inoculation of guinea-pigs practically in all cases in the first four days of the disease, thereafter it gradually disappears, and in the second week, when jaundice is most marked, it cannot be detected. The spirochæte is sometimes present in the blood in the human subject in numbers sufficient to allow its detection by direct microscopic examination at the early stage. Taylor and Goble in the Andamans had success with blood cultures when carried out between the third to the sixth day of illness. The spirochæte may be detected in the cerebro-spinal fluid after it has disappeared from the blood.

Of the internal organs the liver contains the organisms in largest numbers, they may be found also in the suprarenals, and, especially at a later stage, in the kidneys. In all the organs in the human subject at post-mortem examination the leptospiræ are scanty, they are often somewhat irregular and degenerate in appearance, and occur in the interior of the special cells as well as in the interstitial tissue. These facts have been explained as being the result of the formation of antibodies, which drive them from the blood into the interstitial tissues, thus they may be found in fibrous tissue of the wall of the aorta. Their late occurrence and persistence for some time in the kidneys are comparable with what occurs in the natural infection of the rat without the occurrence of disease symptoms (*vide infra*). The organism is also excreted in the urine. This does not occur in the earliest stage of the disease, but from about the eighth to the twenty-first day, positive results are obtained in at least a fifth of the cases. Thereafter it gradually disappears and is rarely found after the fortieth day. The presence of the leptospira in the urine is best demonstrated by inoculating a guinea-pig. As the organism is easily damaged by an acid or alkaline reaction and by serum antibodies special precautions must be taken in the examination (*vide infra*). Microscopic examination of the centrifuged sediment is less satisfactory.

Experimental Inoculation. The injection of blood or of organs containing virulent leptospiræ into a pig leads to the same disease as the subcutaneous injection of the organisms.

The symptoms are jaundice, tendency to hæmorrhages, albuminuria, and anæmia. There is pyrexia, which towards the end is normal temperature; the fever usually appears in the period of the third or fourth day and usually appears in the period of the fourth day of the illness and

Post mortem, the subcutaneous hæmorrhages may be generalised

as they may be confined to the lungs, intestinal walls, and retro-peritoneal tissue. acute parenchymatous or hæmorrhagic nephritis is present, and the spleen is large and congested. The lungs show hæmorrhages as small and large spots, being described as 'like the wings of a mottled butterfly'. The leptospiræ are present in the blood and organs, and in the latter are chiefly interstitial in position, few being actually within cells. In this respect there is a difference from what obtains in the human disease. They are most abundant in the liver, where they may be arranged like a 'garland' round the liver cells. The adrenals and the kidneys contain considerable numbers, but they are scanty in the spleen, bone marrow, and lymphatic glands. Young guinea-pigs are much more susceptible than older animals. The disease may be raised by further passages. The loss of virulence of the organisms in urine is attributed to the acidity or to the presence of bile or serum antibodies, and microscopically they may appear degenerate and atypical. Other animals, such as mice, although little susceptible to inoculation with the organism may show marked production of agglutinins.

The invasive property of the leptospira is of value for isolating it from impure cultures. 0.5 to 1.0 c.c. of culture is injected intraperitoneally into a guinea-pig and ten minutes later a similar amount of blood is withdrawn from the heart and used to inoculate several tubes of culture medium. *L. biflexa* (p. 509) can be recovered similarly (Schuffner).

Source and Mode of Infection. As regards the route by which the infection enters the body, the Japanese workers believed that in the human disease infection occurs chiefly through the alimentary tract, and they were able to infect guinea-pigs by feeding with material containing the organism or by introducing some of it into the rectum. Buchanan and others, however, failed to produce infection in animals by feeding. The organisms are very susceptible to acid and are probably often killed by the gastric juice when swallowed. But an epidemic of the disease in Lisbon was traced to infected drinking water (Jorge). The Japanese observers showed that infection could take place through the apparently intact skin, and found that this occurred with comparative rapidity, as the application of an antiseptic five minutes after the infective material did not prevent infection. The occurrence of infection through the skin has been confirmed experimentally by Buchanan and others. This agrees with clinical observations on the development of the disease after immersion in polluted waters. This mode of infection has been utilised for demonstrating the presence of the organism in contaminated

water by bathing with it the scarified skin of a guinea-pig (Appelman *et al.*)

A highly important point with regard to the epidemiology of the disease is the common presence of the leptospira in both house and field rats and

relatively high. The organisms are practically confined to the kidneys, and we have here a resemblance to what is found in the human infection at a later stage when antibodies are present in the blood. The leptospiræ are passed in large numbers in the urine of the infected animals, and in this way contamination of the soil and various articles is brought about. Thus, apart from cases due to immersion in contaminated water, leptospirosis in civilians is an occupational disease which afflicts miners, farm-workers, sewer-men, and fish-workers principally. The organisms obtained from rats are stated to vary considerably in virulence for experimental animals, this may be due to attenuation by the acid of the urine (*vide supra*). Buchanan demonstrated

infective jaundice may exist as a saprophyte under natural conditions, and from a natural source infect animals or man (*vide infra*).

It should be noted here that an infectious jaundice of dogs, especially young animals, known as 'yellows', has been shown to be due to *L. icterohæmorrhagiæ*, and the condition corresponds to the human disease. The infection has also been observed in a fox.

Immunity. The development of antibodies in the patient's blood occurs during the disease. These appear toward the end of the first week, and seem to be related to the disappearance of the organism from the blood; they become marked during the second week. Their presence can be demonstrated by spirochætoidal, lytic, agglutination, complement-fixation, and adhesion reactions. Spirochætoidal action is detected by injecting some of the patient's serum along with the organisms into a guinea-pig; lysis also occurs, corresponding to Pfeiffer's phenomenon in the case of bacteria. The demonstration of agglutinins in the patient's serum is of great value in diagnosis, as they reach a high titre and persist for years afterwards (*vide infra*). After three to four weeks the titre often reaches levels of 1 in 10,000 to 30,000, the maximum being between the thirtieth and fiftieth days. A marked rise of titre between the tenth and fourteenth days is significant. A negative reaction at the end of the fourth week of illness practically excludes this disease. But as there is marked serological specificity shown by different species of pathogenic leptospiræ, this must be considered before deciding that a case is not due to leptospiral infection. A phenomenon which must be borne in mind is that a serum which agglutinates the living leptospiræ in higher concentrations may cause lysis when more dilute. This lytic effect is shown by heated sera and so is independent of complement. Rats which are carriers of the leptospira also have antibodies in their serum.

Specific antisera may be developed by injecting animals, e.g. rabbits, repeatedly with killed or living cultures. By agglutination with such antisera *L. icterohæmorrhagiæ*, as met with in Europe, has been shown to be practically uniform serologically; it contains two antigenic components, one of which may be deficient in certain strains. There exist, however, a number of leptospiræ which are serologically distinct (*vide infra*). The adhesion

reaction has also been utilised by Brown and Davis in the identification of leptospiræ. The results are analogous to those of agglutination tests with immune sera, but are stated to be more quickly obtained and more easily observed.

Serum therapy and prophylactic immunisation Therapeutic antisera to the *L. icterohæmorrhagiæ*, with properties similar to those observed in the serum during the disease (*vide supra*), have been prepared and have proved of value in the treatment of cases. Experimental results indicate that the antiserum should be given as soon as possible. Also, effective protection by the use of a vaccine consisting of killed cultures of the organism has been recorded (Want).

Bacteriological Diagnosis. *Demonstration of organisms in the blood.* In the first week of the disease in a proportion of cases the leptospira may be seen on direct examination of a wet film of blood by dark-ground illumination. Kligler and Ashner centrifuged the citrated blood at 1,000 to 1,500 revolutions per minute for five to ten minutes, then separated the supernatant fluid and

toneal cavity of a young guinea-pig (not over 175 grms in weight). The leptospira may survive for some days in clotted human blood; if this is the only material available, it should be ground up and used for inoculation. With a virulent infection the animal becomes jaundiced and dies in seven to fourteen days. Leptospiræ can then be found in the liver, lung, and kidney either by dark-ground examination of emulsions of the organs or in sections of the tissues treated by Levaditi's method. Older guinea-pigs or more resistant animals may not become ill, to obviate this difficulty the animal's temperature should be recorded daily and when a sustained or intermittent pyrexia occurs a specimen of fluid should be removed from the peritoneal cavity for both microscopic examination and inoculation of a fresh guinea-pig. Several days later the heart blood should be examined. Repeated passages tend to increase the virulence of the organism for the guinea-pig. It is advisable in any case not to wait for death of the animal before making a passage, as the virulence of the leptospiræ is diminished at this stage. Positive blood cultures may be obtained by inoculating four or five tubes of medium, each with 0.1 to 1 c.c. of the patient's blood and incubating for seven to twenty-one days. The cerebro-spinal fluid may yield positive results after the organisms have disappeared from the blood.

Urine Especially from the twelfth to the eighteenth day of illness positive results are likely to be obtained by repeated inoculation of guinea-pigs with urinary sediment. Since an acid or alkaline reaction harms the vitality of the organisms, it is necessary to secure a nearly neutral urine, e.g. by administration of alkalis to the patient, and to adjust the reaction of the specimen to neutral without delay, also, the animal should be inoculated within one hour of the urine being passed, receiving the sediment of 60 to 80 c.c. of urine after centrifuging for fifteen minutes.

Agglutination reaction with blood serum Well-grown cultures may be used in the living state or immediately after addition of formalin (0.2 to 0.5 per cent. of a pure 40 per cent. solution of formaldehyde), as the agglutinable suspension. Mixtures of the culture are made with a wide range of dilutions

volume of six drops in each tube; and distilled water is added

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normal saline. With a formolised culture the same method may be used or mixtures of 0.6 c.c. of the culture are made with 0.2 c.c. of doubling dilutions of the patient's serum from 1 in 2 onward, to yield final dilutions of 1 in 8, 1 in 16, 1 in 32, etc. The tubes are kept at 30° to 37° C. and the results are read after three, and twenty-four, hours. With living cultures readings are best

complete agglutination is recognised by the formation of a fluffy deposit visible to the naked eye. With living cultures and microscopic readings, titres of 1 in 30 or upwards are considered positive, but lower titres are often significant. With formolised cultures especially if read macroscopically, some workers consider that to be significant the titre should be over 1 in 100. If formolised cultures are used which have been kept for a considerable period, then agglutinability should be controlled frequently by the use of a dried standard serum (see Gardner).

As a positive serum reaction may not develop until after the organisms
 1 day)
 anti-
 titre

of agglutinins on repeated examinations from the tenth to the fourteenth day. If the patient has received a therapeutic injection of anti-leptospiral serum seventy-two hours or longer before the test, validity of the agglutination reaction will not be affected (Alston).

Isolation of L. icterohæmorrhagiæ from contaminated water. An area of the abdominal skin of a small guinea-pig is shaved (without the use of soap) and a series of scarifications made about 8 cm. long and 0.5 cm. apart. The area is then immersed for one hour in the water, which is kept at 30° C. Afterwards the animal is placed in a warm cage until dry (about five hours).

Demonstration of the infection in rats. Scrapings of the kidney cortex should be examined microscopically by the dark-ground method, both organs being examined because of irregular distribution of the leptospire. Sections should also be prepared by Levaditi's method. The most constant evidence is the presence of agglutinins in the serum, titres of 1 in 100 and upward being taken as diagnostic of present or past infection (Larson). Also guinea-pigs may be inoculated with an emulsion of the rat's kidney or urine, but this method succeeds only when the animal is newly dead (Smith).

OTHER PATHOGENIC LEPTOSPIRÆ

A number of leptospira, all microscopically indistinguishable from *L. icterohæmorrhagiæ* have now been recovered from man or animals which differ serologically from that organism and also among themselves as shown by direct agglutination and the agglutinin-absorption reaction (Walch-Sorgdrager). However, they are more or less related as regards their antigens. The clinical conditions are often like influenza or mild typhus fever and many of them are relatively mild, but in some jaundice occurs along with considerable mortality. The virulence for man is to a great extent paralleled by that for the guinea-pig. Enlargement of the lymphatic glands and rashes are features of certain of the diseases. The species of animals which are affected

or act as carriers vary also. These infections are proportionally more frequent in man in countries outside Europe, having been met with in various parts of Asia—Japan, Dutch East Indies, Andaman Isles—and in Australia. On the continent of Europe *L. canicola* affects dogs, especially in Holland, and also occurs in Great Britain; man is occasionally infected. Swamp-fever or mud-fever, met with in man in various parts of the continent of Europe, is caused by *L. grippo-typhosa* and cases in Denmark and elsewhere are due to infection with *L. sejroe*. Leptospirosis of cattle has been found in Russia and Palestine (see Bernkopf *et al*), as well as Australia. Diagnosis of the conditions is usually made by the agglutination reaction; but there is reason to believe that after recovery from some of them antibodies disappear from the serum more rapidly than in cases caused by *L. icterohæmorrhagiæ*. What has been stated about the latter as regards cultivation, use of guinea-pigs for recovering the organism, etc., applies generally to those other pathogenic leptospiræ.

L. canicola, found by Schüffner and Klarenbeek in Holland, affects dogs, causing uræmic symptoms with little tendency to jaundice. It occurs also in Great Britain and the United States of America, and in Germany may be the cause of the 'Stuttgart disease'. Rats do not harbour this organism; thus the occasional cases in man are usually contracted directly from the dog (see Baber and Stuart).

Leptospira grippo-typhosa of Tarasoff (*L. andaman B*) has been recovered from the blood in cases of swamp-fever or mud-fever, a condition which occurs in epidemics among workers on the land in the warm season after extensive flooding in Eastern Germany, Russia, France, Italy, Switzerland, and in the Andaman Isles. In this disease there is a sharp febrile attack which lasts less than a week and the mortality is low; jaundice is not a feature. There may be one or several relapses. The organism has little virulence for guinea-pigs or mice, and the best method of demonstrating it is by means of blood culture during the first two days of the fever; it is also present in the blood before the onset of fever. The chief animal carrier is a field vole, *Microtus arvalis*. Although no cases have been observed in Great Britain, certain species of rodents indigenous here have occasionally been found infected on the Continent. The disease has been produced in general paralytics for therapeutic purposes by injecting a pure culture of the leptospira.

L. sejroe (Petersen and Christensen) was first found in the Danish island of Sejrøe; it is the cause of outbreaks usually of mild disease. The infection has also been contracted in Italy and other parts of Europe. The carriers are

L. hebdomadis. This is a disease of low fatality met with in country districts in Japan in autumn. Jaundice is rare, but there is enlargement of lymphatic glands. Ido, Ito and Wani established the constant presence in the disease of *L. hebdomadis*. The leptospira is discharged in the urine, often in large numbers, and especially in convalescence. It is only slightly pathogenic to guinea-pigs. *L. hebdomadis* is harboured by the wild mouse, *Microtus montebellii*, and the organisms are present in the kidneys and excreted in the urine of these animals.

L. autumnalis (*L. akiyami A*) produces in the autumn an endemic infection in a country district of Nagasaki province, Japan. Mild jaundice is frequent, death seldom occurs. Opacity of the vitreous is usual. The carriers are

L. rachmat, *L. salnem* (or *pyrogena*),
infections in the Dutch East Indies, which resemble Weil's disease; the

mortality is moderate. Plantation workers are affected especially. Guinea-pigs are fairly susceptible to these leptospiræ and there is often jaundice. The two latter are carried by rats. Cats have been found to carry *L. bataviæ* and other species (Esseveld *et al.*). *L. bataviæ* (*mitis*) has also been found in rice-field workers in Italy.

L. andaman A produces a form of Weil's disease in the Andaman Isles. *Leptospira* is fairly viru-

stal fever' in North Queensland. The disease is severe, but not very fatal. Lymphadenitis is common, but jaundice rare. These organisms are carried by the cane-field rat, *R. culmorum*. Guinea-pigs are fairly susceptible to the infection.

L. pomona and *L. mitis* (*bataviæ*) both produce a 'seven-day fever' type of illness, rarely accompanied by jaundice or fatal, among dairy workers in Queensland. The infections are apparently endemic among pigs and cattle. The rodent carriers are not known. Guinea-pigs are not severely affected. *L. pomona* has recently been recognised as the cause of disease in swineherds in Switzerland.

LEPTOSPIRA BIFLEXA

Non-pathogenic leptospiræ have been observed in water supplies, mines, etc., by various workers (Fig. 148). They are especially prevalent where there is a slimy deposit, e.g. at the mouth of taps. This type of organism can be readily cultivated like the other leptospiræ, but is very sensitive to salt, also to acid. It can be demonstrated by enrichment, e.g. in London tap water, by adding about 1 per cent. sterile faeces and keeping the mixture in a Petri plate at 25° to 30° C. for ten days to three or four weeks (Hindle). Pure cultures may be obtained by filtration through a Berkefeld filter or by Schöffner's method. It has been claimed that by passage in animals it may become virulent and capable of causing in guinea-pigs the same pathological condition as *L. sclero hæmorrhagica*. Also, since the latter may survive for a time in wet soil, water, etc., it has been suggested that the saprophytic leptospira is merely a modified form of the pathogenic variety (Bärmann and Zuelzer), but there is no clear proof of this. Strains of *L. biflexa* are serologically distinct from the pathogenic leptospira; also they differ among themselves.



FIG. 148. *Leptospira biflexa*, as seen in a culture obtained from water, showing elementary spirals. Kirkpatrick's stain. 1,200.

SPIRILLUM MINUS (SPIROCHÆTA MORSUS MURIS)

One form of rat bite fever (Sodoku), following the bite of a rat after an incubation period usually of seven to twenty-one days, presents a characteristic clinical syndrome— inflammation and ulceration of the skin in the neighbourhood of the bite, which may have completely healed by the time of onset of the disease, paroxysms of fever of the relapsing type, swelling of lymph glands and a patchy erythematous skin eruption. In untreated cases the infection may persist indefinitely. It responds to salvarsan therapy. The disease has been observed in various parts of the world but has been most

studied in Japan, and in 1915 Futaki and his co-workers described a special type of spiral organism in the skin lesion and in the lymph glands, which has now been established to be the specific causal agent of the disease. They regarded it as a spirochæte and it was designated *Spirochæta morsus muris*; it is now called *Spirillum minus*. (The other form of rat-bite fever is dealt with on p 470.)

MICROSCOPIC CHARACTERS. The organism is comparatively short, measuring 2 to 5 μ , and presents a few regular curves of 1 μ each (Fig. 149); it is relatively thick and easily stained. By dark-ground illumination the blood or peritoneal fluid of an infected mouse shows a 'darting' type of motility of a vibrio. Polar flagella (1 to 7) are present at each end; they are more frequently multiple than single.



FIG. 149. *Spirillum minus* from a naturally infected mouse, stained (McCluskie).

The organism is practically rigid. Thus it has been considered that its biological characters conform more to those of a spirillum than a spirochæte.

The flagella are difficult to stain, they may be demonstrated by fixing very thin films, immediately after spreading and before drying has occurred, for thirty to sixty seconds over the vapour of 1 to 2 per cent. osmic acid solution and then, when dry, staining overnight in a 1 : 10 or 1 : 15 dilution of Giemsa's solution.

CULTIVATION. Futaki and co-workers obtained growths in Shimamine's medium (sodium nucleate dissolved in horse serum, carbon dioxide passed through the solution, and the medium heated at 60° to 65° C.) Other observers have failed to obtain cultures, however, in various media, and the Japanese workers were unable to maintain their growths after subculture.

Relations to the Disease, Experimental Inoculation, and Immunity. The causal organism occurs in the local lesions, the related lymph glands, and in some cases in the blood. It has now been demonstrated in a few cases of rat-bite fever in this country, including one in Scotland (Mackie). It is of interest that a condition similar to rat-bite fever has followed the bites of cats, dogs, and ferrets.

Rats, mice, and guinea-pigs can be infected with the spirillum of rat-bite fever, and the organism appears in their peripheral blood. Its virulence to laboratory animals is relatively low, though guinea-pigs are fairly susceptible and, after a time, succumb to the infection. Occasionally individual guinea-pigs are resistant. Mice show no symptoms when infected; they usually become refractory to reinfection after the initial attack, and the organisms practically disappear from the blood, although they persist

in larger numbers in the peritoneal cavity (Saisawa and Taise). The disease has been produced in the human subject by injecting blood of infected animals. The spirillum occurs in a small percentage of rats and field mice under natural conditions, and the disease has been produced in the guinea-pig from the bite of an infected rat. The mechanism of infection is doubtful. It has been suggested that when the animal bites, bleeding from the mouth provides the material for inoculation. Infected animals may develop a keratitis with spirilla in the conjunctival secretion, and from this the nasal and mouth secretions become infected. It has been shown also that antibodies develop in the blood of convalescent patients and infected animals, these possess protective and immobilising properties, etc. In a proportion of patients the blood may give positive Wassermann and flocculation reactions.

An organism morphologically identical with *Sp. minus* is sometimes found in the blood of mice and the infection can be passed indefinitely through fresh animals, but it does not flourish in other species. It is non-pathogenic to mice and may also be non-infective to man (McCluskie). Similar spirilla occur in rats and guinea-pigs. It is necessary to ensure beforehand that animals used for experimental inoculation are free from such natural infections.

Bacteriological Diagnosis. Excision of an enlarged gland and inoculation of several mice (or rats) and guinea-pigs intraperitoneally, subcutaneously, and intracutaneously with the emulsified tissue provides the most certain method of demonstrating the spirillum, or the gland may be punctured with a syringe and fluid aspirated, which is used for inoculation. In some cases the organisms may be demonstrated directly in the local lesion, and the exudate from this may be used for animal inoculation. Also, 3 to 5 c.c. of blood withdrawn during the pyrexial stage can be injected intraperitoneally into a guinea-pig, but this method may fail. After a varying incubation period up to a month for mice—the spirillum can be demonstrated by the methods mentioned above, in the peripheral blood of the infected animals.

CHAPTER XXVI

THE RICKETTSIAE OF TYPHUS FEVER, ETC.: BARTONELLA

The term *Rickettsia* was originally applied to certain definite structures found to develop in the alimentary tract of lice after feeding on cases of typhus fever ('typhus exanthematicus'). It has also been shown that the disease is transmitted by inoculating susceptible animals with the faeces of insects containing these 'rickettsia bodies'. Similar bodies occur in the cells of the lesions in man and experimental animals. Several other diseases have likewise been found to be transmitted by arthropods harbouring such bodies, namely, 'murine' typhus by the rat flea, Rocky Mountain Spotted Fever by ticks, another typhus-like disease by mites, and trench fever by lice, also the South African disease of cattle known as 'heart-water'. The name *Rickettsia* commemorates Ricketts, who investigated those diseases and died of an attack. The rickettsia bodies are very minute diplococcal or rod-shaped structures, measuring as a rule less than $1\ \mu$, although longer forms occur and considerable pleomorphism is shown. They are Gram-negative and have only a weak affinity for the ordinary bacterial stains. In order to demonstrate them Giemsa's solution has been much used; with this they stain a blue to reddish or purple colour. Macchiavelli's method is also suitable for smear preparations. The finding of rickettsiae has led to much investigation regarding their occurrence in various arthropods and their significance. On the one hand, the specific etiological relationship of the rickettsia bodies to the respective diseases appears, in the case of lice, from the fact that insects fed upon healthy persons do not develop them and that their appearance in the gut coincides with the acquisition of infectivity by the lice. On the other hand, similar bodies have been found in various blood-sucking arthropods apart from the occurrence of disease in their mammalian hosts. Thus Nöller showed that *Melophagus ovinus* (the sheep 'ked') habitually harbours rickettsiae, the bed-bug (*Cimex lectularius*) is constantly infected with such bodies (Arkwright, Atkin and Bacot), and they occur in the ovaries of the mosquito, *Culex pipiens* (Hertig). Their presence has also been demonstrated in a number of other arthropods by Hertig and Wolbach, and others. As regards the nature of the rickettsia bodies there has been some uncertainty, although it is clear that they are definite structures distinguishable from mitochondria, etc., that they are living objects capable of multiplication seems evident. In the case of typhus fever they appear in large numbers in the intestinal contents of the lice after some days following the ingestion of infected blood, and also they are seen to pack the epithelial cells of the gut and to cause disturbances in function which lead to the death of the insect. Other rickettsiae again are harmless to the arthropod hosts in whose tissues they appear to flourish in permanent symbiosis, being transmitted in the eggs from one generation to another. In general, attempts have failed to obtain cultures of pathogenic rickettsia bodies on the usual artificial media, although they have been found to multiply in tissue cultures, in media containing minced fresh tissues, and especially well in the yolk sac of the developing fowl's egg. That is to say, they behave mostly as obligate intracellular parasites; however, they grow best in living cells which are not proliferating actively. But, *Rickettsia melophagi* grows on blood agar and in its behaviour

in culture resembles the ordinary bacteria. Most pathogenic rickettsiae do not pass filters which retain the ordinary bacteria (according to Pinkerton this may be due to their low viability when separated from tissue cells). With the electron microscope their appearance resembles that of vaccinia virus and ordinary bacteria in that they have a limiting membrane surrounding a substance apparently of protoplasmic nature (Plotz *et al.*). Accordingly their general characters would place the rickettsiae in an indefinite group intermediate between bacteria and viruses. No very satisfactory classification is available, apart from one based on the species of arthropods which harbour the various rickettsiae, that adopted for the forms pathogenic to man rests on immunity reactions. Thus recovery of an infected animal is followed by insusceptibility to reinfection only with the same species of rickettsia, also there is the development in the serum of the infected individual of agglutinins specific for the causal organism, as well as of complement-fixing antibodies and of anti-infective substances. These reactions are fairly closely paralleled by the Weil-Felix reaction, namely the agglutination of particular members of a series of standard strains of *B. proteus* by the serum of infected subjects.

Classical typhus fever in a moderately severe attack is characterised by pyrexia, which quickly reaches a high level and is maintained for nearly a fortnight, at the onset severe headache with mental disturbance passing later into delirium, and a characteristic skin eruption. The mortality is low in childhood, but after the age of thirty years it increases to upwards of 50 per cent. The other rickettsial diseases are clinically similar, but all show much variation in severity, in some a local lesion tends to develop at the site of inoculation. This group of diseases is very highly infective for man and elaborate precautions must be taken against spread, especially through the air, among susceptible individuals working with the rickettsiae (van den Ende *et al.*)

RICKETTSIA PROWAZEKI (OF LOUSE-BORNE TYPHUS FEVER)

In the following description the form of typhus fever will be referred to which has long been known to cause serious epidemics in Europe and Asia. Since it has been learned that similar diseases exist which can be more or less clearly distinguished, this original type has been variously named—epidemic 'Old World', 'classical', 'louse-borne', 'human'. The essential feature of this infection is its transmission from man to man through the agency of lice and the organism has maintained high virulence, presumably as a result of constant passage through one susceptible species of mammalian host. An attenuated form of the disease may exist, however, *e.g.* the condition known as Brill's disease met with among certain European immigrants in New York and other towns of the north-east coast of the United States.

The first definite results regarding the etiology of typhus fever were obtained by Nicolle in 1909. This observer found that the blood of cases of typhus fever during the febrile period, as well as immediately before and afterwards, was infective for chimpanzees. The blood is highly infective during the two weeks of acute illness. A febrile illness which was practically identical with the disease in man (including occasionally skin eruption and sometimes fatal) was produced, and the blood of infected animals was again infective towards fresh individuals. A large number of such passages were successful. Some species of lower monkeys are susceptible, especially when inoculated intraperitoneally, but only $\frac{1}{2}$ – $\frac{1}{3}$ of the inoculations result. Of other

animals the most susceptible to the infection is the guinea-pig, in which, following intraperitoneal inoculation with several c.c. of infective blood, there occurs an illness after an incubation period, which is seven to twelve days as a rule, characterised by fever and loss of weight; the illness lasts for four to eleven days and is only exceptionally fatal. In the guinea-pig the infective agent is present during the febrile period in the blood and solid organs especially the brain, and in the tunica vaginalis of males. In this animal likewise it can be maintained by passage and does not undergo any change in virulence. As in the human subject, the characteristic lesion is a proliferation of the endothelial cells, etc. of the small blood vessels, which form masses in and around the vessels and lead to thrombosis and occlusion. The cellular masses are marked in the brain and in the skin, but are also present in other organs. In the male guinea-pig, after intraperitoneal inoculation an inflammatory exudate may occur into the scrotal sac; but this is much more marked with the murine variety than with louse-borne typhus (*vide infra*). Kusama and Ségal showed that the infective agent is particularly associated with the blood platelets, since a suspension of these elements separated from the other constituents of the blood by centrifuging is specially active, whereas the blood plasma and also leucocytes from peritoneal exudate do not produce the infection. Nicolle found that the infectivity was destroyed by a short exposure of the material at 50° to 55° C.

Rats show no symptoms after inoculation with typhus rickettsiæ, but their blood becomes infective, as can be demonstrated by inoculating guinea-pigs; also agglutinins for the organisms develop. Further, the infection may persist for upwards of a year in the brain. Such a condition is sometimes referred to as 'inapparent infection'. Various other rodents, as well as cats and dogs, can be infected.

The vector of infection. A most important fact established by Nicolle was that under natural conditions infection takes place through the *Pediculus humanus* (corporis variety; probably also *capitis*). Monkeys and guinea-pigs can both be infected by lice previously fed on a human case. Since the louse becomes infective after the seventh day following the infected feed, it is clear that the causal organism undergoes development or multiplication in the insect host. It is present in the *feces* of the lice which constitute the source of infection for man under natural conditions. During the war of 1914-18 serious outbreaks of typhus fever occurred in Serbia, Bulgaria, and Poland, and in the late war at Naples and elsewhere; measures based on the view that the body louse is essential to the spread of the epidemic—especially the use of the insecticide 'D.D.T'—were always successful in controlling its spread. Accordingly, the evidence is practically conclusive that typhus fever is spread from man to man by the louse. It is possible that the causal agent may be transmitted through the eggs of the insect to a second generation of lice, but this appears to be very unusual.

MICROSCOPIC AND BIOLOGICAL CHARACTER. *Rickettsia prowazeki* is the name given by da Rocha-Lima to the small bodies found by himself and Prowazek in the intestine of lice taken from typhus fever patients. These are probably identical with structures described earlier by Ricketts and Wilder (who stated also that they were present in large numbers in the blood of typhus fever patients, but this has not been confirmed), Sergeant, Foley and Vialatte, and others. They are non-motile and are satisfactorily demonstrated by staining with Giemsa's solution, when they appear characteristically as minute cocci, diplococcus-like bodies, or rods measuring 0.25 μ by 0.3 to 1.0 μ , usually of a blue to reddish or purple tint, a pale capsule has also been described round the ends (Fig. 150). There is considerable

pleomorphism, and thicker forms, threads and chains up to 4 to 10 μ or even 20 to 50 μ are also met with

The rickettsiæ appear after an interval of about a week or longer in a

the alimentary tract of the lice from the mid-gut downward. The infected cells become distended with the bodies, so that the nutrition of the infected insects is interfered with and they die. The rickettsiæ are also found in enormous numbers in the faeces of infected lice owing to rupture of the distended cells.

The transmission of typhus infection to guinea-pigs is effected by inoculation with the gut contents of lice harbouring the rickettsia bodies, and in the absence of these bodies the guinea-pigs do not become infected. Also, blood platelets of infected guinea-pigs when injected *per anum* into lice cause the appearance in the latter of *R. prowazeki*, as shown by Bacot and Ségat. The excreta of infected lice remain virulent as a dry powder at room temperature for at least eleven days and probably for over two months (Starzyk). The high infectivity of such material would explain the frequency with which this disease has been contracted by investigators in the laboratory (Arkwright and Bacot). It appears that the entrance of infected louse excreta into minute punctures of the skin, *e.g.* the bites of the insects and scratches, constitutes the chief mode of infection in the human subject under natural conditions. But there is evidence also that the rickettsiæ may be inhaled and enter the body through the mucous membrane of the nose and respiratory tract. When a suspension of virulent rickettsiæ, *e.g.* in guinea-pig's brain, is introduced into the nose of mice and cotton rats, a pneumonic lesion develops. By using a strain of organisms which has been repeatedly passed in this way mice die in forty-eight to seventy-two hours and very great numbers of rickettsiæ are found in the lungs (Castaneda, Andrewes *et al.*). The infectivity of rickettsiæ is rapidly destroyed by heating at 60° C. and by antiseptics. Suspensions stored in serum broth and frozen at -77° C. remain alive and virulent for many months (Elford *et al.*).

(A rickettsia which is also situated within the epithelial cells of the intestine in lice has been met with occasionally and was named *R. rocha-lima* by Weigl.

man, or
by conta :

it is a variant of the latter or a separate species is not determined—see Sparrow.)

The rickettsia bodies may also be demonstrated in the endothelial cells of the vascular lesions of the skin, brain, and other organs from human cases and, though less frequently, in the tissues of infected guinea-pigs (Wolbach *et al.*). The great numbers found in the lungs of mice when the infection has been accommodated to these animals, have been utilised as a source of vaccine.

Macchiavello's method of demonstrating Rickettsiæ. A smear of tissue is made on a microscope slide, allowed to dry in the air and then gently warmed. On to this is filtered, through coarse paper, 0.2% per cent. solution of basic fuchsin in distilled water



FIG. 150. *Rickettsia prowazeki* from teased gut of an infected louse. An intestinal epithelial cell is seen containing numerous Rickettsia bodies. Giemsa's stain $\times 1,500$. (From a specimen lent by the late Sir J. A. Arkwright.)

brought to a pH of 7·2 to 7·4 with NaOH or Na_2CO_3 . The stain is allowed to act for four minutes and is then washed off very rapidly with 0·5 per cent citric acid solution and again very rapidly washed with tap water. The film is then stained with 1 per cent aqueous methylene blue for about ten seconds. Both intra- and extra cellular rickettsiæ stain red; cells are blue.

Tissue, e.g. brain, which has been fixed in formalin, is cut into slices 2 to 3 mm. thick, washed in running water for twenty-four hours, and then stained for thirty-six hours with Giemsa's solution. Differentiation is best effected by exposure of the stained sections to strong sunlight; the rickettsiæ then appear of a deep bluish purple tint, while the cytoplasm of endothelial cells is faint blue. The methods of Castaneda and Nyka may also be used for demonstrating the bodies (Appendix).

Thereafter the tissue is washed for twenty-four hours, dehydrated, etc., and embedded in paraffin. Sections are stained with Giemsa's solution overnight. Differentiation is best effected by exposure of the stained sections to strong sunlight; the rickettsiæ then appear of a deep bluish purple tint, while the cytoplasm of endothelial cells is faint blue. The methods of Castaneda and Nyka may also be used for demonstrating the bodies (Appendix).

CULTIVATION Although, as has been noted, no growth is obtained on any of the ordinary culture media for bacteria, proliferation of the rickettsiæ occurs in surviving susceptible cells. Wolbach and Schlesinger found that *R. prowazeki* multiplied in the endothelial cells in tissue cultures and material from a 'subculture' twenty-eight days after removal from the animal was infective when inoculated into a fresh guinea-pig. Nigg and Landsteiner used a medium of Tyrode solution, serum and minced tissue similar to that of Matland for vaccinia virus, and effected six passages. Zinsser *et al.* obtained cultures as follows: the tunica vaginalis of a guinea-pig six to seven days after intraperitoneal inoculation is finely minced with a few drops of serum and Tyrode solution and the mixture spread on the surface of a sloped tube of agar, and kept for six to ten days. The rickettsiæ multiply in the chorio-allantoic membrane of fertile hens' eggs. Cox's discovery that after inoculation direct into the yolk sac very great numbers of the bodies accumulate and death of the embryo occurs has proved of the greatest value for the preparation of vaccines. As the inoculum, the brain or tunica vaginalis of an infected guinea-pig during the period of fever, is used.

Immunity. Nicolle observed that, as in the case of man, when an experimental animal has passed through an attack of the disease it becomes immune to reinoculation. By taking advantage of this fact it has been shown in cross-inoculation experiments that louse-borne typhus fever as met with in different parts of the world is immunologically the same. It had been known that children under ten years are less susceptible to typhus than older individuals, and Nicolle made the interesting observation that when a family is attacked, young children, while apparently well, may really suffer from a slight rise of temperature. This condition represents an abortive attack of the fever as the blood in such cases is infective for animals. These abortive cases may play a part in the dissemination of the disease. Nicolle's results have been confirmed in America. According to Zinsser, Brill's disease represents a recrudescence in individuals who are the subjects of an inapparent infection following an attack of typhus fever in childhood.

The serum of both man and animals during convalescence confers passive

serum are more persistent

Dietrich—show a high

Accordingly, the agglutination reaction is of great value. In louse-borne typhus from murine, the same is true of the complement-fixation reaction. Nicolle and Conseil, by injecting into asses intravenously or intracerebrally emulsions of the brains of infected guinea-pigs, produced an antiserum which had protective effects in guinea-pigs. Successful

attempts to produce effective antisera by means of killed rickettsiæ have been recorded by Zinsser and Castaneda. They injected into a horse large amounts of rickettsia bodies (derived from the tissues of rodents infected with the murine form of the disease) treated with formalin. The resulting antiserum, when injected into guinea-pigs several days after inoculation with European typhus material, prevented development of the disease. Antisera may protect against rickettsial toxins (Report, 1916).

Active immunisation has been effected in the human subject by injecting several small doses (1 c c) of serum derived from cases of typhus fever or of infected guinea-pigs' serum or tissues at the height of the fever, but this procedure is not safe as a general measure. Weigl has used as a vaccine for the human subject the intestinal contents of lice infected with the typhus rickettsia, which are ground up in a weak solution of phenol. Although this method has proved effective, it is not adapted for preparing considerable amounts of vaccine, since the large numbers of lice required must be fed on human subjects who must be already immune to typhus fever. Two types of vaccine have been widely used—(a) a formalised suspension obtained from the lungs of mice infected intranasally with an adapted strain of the organism (Durand and Giroud) and (b) a suspension of the rickettsiæ grown in the yolk sac of developing chick embryos and treated with phenol and formalin (Cox and Bell). The rickettsiæ are freed from other constituents by differential centrifuging. Several injections are given and should be repeated yearly. These vaccines do not cause any severe effects, but they may not prevent a subsequent attack. In human subjects so vaccinated and then inoculated with virulent organisms six to eight weeks later the incidence of the disease was unaffected, but the mortality was abolished, according to a report of Ding in Germany. Cox and Bell were able to protect guinea-pigs against infection with *R. prowazeki* by means of their vaccine.

Weil-Felix reaction. The tendency for the blood serum in typhus fever to contain heterologous agglutinins was originally observed by Wilson, who found a positive Widal reaction for *B. typhosus* in such cases. Bacilli of the *B. proteus* group have been cultivated by Weil and Felix from the urine and blood of typhus fever cases and also *post mortem* from the organs, which, while certainly not the causal organisms, are important because of their being powerfully agglutinated by the blood serum of typhus fever patients. The strain designated *B. proteus* OX-19 is constantly agglutinated in high dilutions, while it is not affected by serum from almost any other condition, except Brucellosis (see Findlay), thus the reaction possesses great value in diagnosis. The agglutinins for this organism appear in the blood on the fourth or fifth day of the disease in about 75 per cent. of cases and reach their height in the second week, the titre may be 1 in 5,000 or even 1 in 100,000. They may still be present many months after convalescence, but the reaction may become negative in from six weeks to three months after the onset of the illness. The explanation of the Weil-Felix reaction according to Castaneda is that the rickettsia and the *Proteus* bacillus possess a common carbohydrate antigen. Rabbits injected with typhus rickettsiæ develop agglutinins for *B. proteus* OX-19, but *agglutinated* t. Another strain of *B. proteus* which is strongly, by the serum of typhus fever

agglutinated (For details of the test and its use in diagnosis, *vide infra*). According to Felix, the antigen of *B. proteus* OX-19 corresponds to the heat-stable antigen of *R. prowazeki*, while the thermolabile antigen of the rickettsia resembles the Vi antigen of the typhoid bacillus (see Craigie *et al.*)

RICKETTSIA MOOSERI (OF FLEA-BORNE OR MURINE TYPHUS FEVER)

Neill (1917) and Mooser showed that male guinea-pigs inoculated intraperitoneally as 'tabardill

was usually absent in animals infected with the European typhus agent. It has since been discovered that there exists both in the eastern and western hemispheres a disease closely resembling typhus, but which is relatively mild, does not tend to occur in epidemics, and is not spread by lice. It is known by various names in different localities, e.g. in Malaya, urban or shop typhus. Further investigation has shown that the rickettsia responsible for this condition is found in the rat flea, *Xenopsylla cheopis* (Dyer *et al.*) and in the tissues (brain) of rats where it may long persist without ill effects (Mooser, Castaneda and Zinsser). Therefore the name *murine typhus* has been given to the disease. When injected intraperitoneally into male guinea-pigs this rickettsia causes after four to six days a very marked inflammatory swelling of the scrotum. The exudate in the tunica vaginalis shows numerous rickettsia bodies (*R. mooseri*) in the endothelial cells, which are microscopically indistinguishable from *R. prowazeki*. The localisation at this site is attributed to its lower temperature being more favourable to the growth of the organisms. The Weil-Felix reaction with the different strains of *B. proteus* is the same as for louse-borne typhus fever, i.e. strong agglutinins develop in patients' serum for *B. proteus* OX-19; and *R. mooseri* is agglutinated. It is noteworthy, however, that while *R. prowazeki* is agglutinated also, the titre for the latter is much lower. Similarly, the serum from cases of louse-borne typhus shows a higher titre for the homologous rickettsia than for *R. mooseri*. There is also a considerable degree of cross-immunity between this infection and that of classical typhus. On reducing the resistance of guinea-pigs or rats by exposure to X-rays or other means, very large accumulations of *R. mooseri* can be got in the peritoneal cavity; and these killed with formalin may be used as a vaccine. On injection of the material into horses, antisera have been obtained which have a curative action in infected guinea-pigs. Growths of the rickettsia bodies may be obtained as in the case of *R. prowazeki*, especially by inoculation of the yolk sac of chick embryos. Rats infected with *R. mooseri* develop a definite febrile illness and may die. Mice inoculated intranasally, e.g. with infected guinea-pig's brain (which is free from other organisms), develop pneumonic lesions which contain abundant rickettsia. The infection apparently exists widely in nature among rats (being spread among these animals by the rat louse, *Polyplox spinulosus*, as well as the flea) and perhaps other rodent fleas is an accidental occurrence.

in man would thus be due to lack of accommodation of the rickettsia in human host. It has not proved possible under experimental conditions to transform the characters of the murine agent permanently into those of the louse-borne variety or vice versa, so far as their behaviour in animals is concerned. But lice can be infected with the murine rickettsia, which is more fatal to them than *R. prowazeki*. The 'Fièvre nautique' of Toulon, *R. mooseri* are present varieties closely related, and it has been shown that they have been impressed by their re-

The antigenic structure of Typhus Rickettsiæ. According to Fulton and Begg, the louse-borne (epidemic) strain and the murine strain, while sharing antigens in common, each possesses a strain-specific antigen as determined by agglutination and complement-fixation reactions with the homologous antiserum. These antigens are regarded as being situated at the surface of the organisms. Boiling a rickettsial suspension deprives it of its strain-specificity; the degraded antigen reacts with an antibody in anti-rickettsial sera (developed in rabbits or guinea-pigs) which is distinct from that which reacts with the undegraded rickettsiæ. The antibody in rickettsial antisera from rabbits which reacts with *B. proteus* OX-19, is a fraction of the antibodies which react with boiled rickettsiæ, but is distinct from the antibodies to the undegraded rickettsial antigens.

DERMACENTROXENUS RICKETTSI (*RICKETTSIA RICKETTSI*) ETC. AND RICKETTSIÆ OF OTHER TICK-BORNE FEVERS

The typhus-like disease, Rocky Mountain Spotted Fever, has been the subject of much investigation in America. It was the first of the group in which the causal agent and the mode of transmission were demonstrated. Its severity varies greatly; in certain districts cases are few but the fatality is high, while elsewhere the disease may be prevalent but mild. The essential pathological anatomy appears to be an inflammatory reaction of the adventitia of the vessels of the subcutaneous tissue and of the genitalia, with degenerative changes in the media, and a perivascular accumulation of large mononuclear cells. There is also thrombosis in the vessels. The disease is transmitted by several species of ticks, *Dermacentor andersoni*, which infests a variety of mammalian hosts is the chief vector to man, while in the Eastern States *D. variabilis* is responsible. Monkeys, rabbits, and guinea-pigs can be infected with the blood of cases, and also by ticks. The natural animal reservoirs have not been discovered, however, although dogs have been found to be naturally infected. The virus is transmitted hereditarily in the eggs of the ticks through consecutive generations. In the guinea-pig the illness is

There is fever with, in
which follows subcutan-
and rash in the ears,
swelling and sometimes necrosis of the feet, etc. However, strains occur
which are of low virulence for guinea-pigs. In the blood vessels in human cases
and in infected guinea-pigs, especially within the large mononuclear cells and
smooth muscle cells, and also in the tissues of the tick, bodies are found which
Wolbach named *Dermacentroxenus rickettsi*, also known as *R. rickettsi*.
These bodies multiply within the endothelial cells in tissue cultures. They
differ from other rickettsiæ in the fact that they invade and multiply pro-
fusely in the nuclei of infected cells, both in the tissues of mammals and ticks
and also in cultures. Some of the bodies closely resemble *R. prowazeki*, but
diplococcal forms occur, especially in the cytoplasm, which may be as large
as pneumococci. Their viability resembles that of the other rickettsiæ.
Abundant growths are got in the yolk sac of chick embryos. None of the
three strains of *B. proteus* hitherto used in the diagnosis of typhus fever is
agglutinated by the serum of Rocky Mountain fever cases to more than a
slight degree. After recovery there is a solid immunity to reinfection, but
there is no cross immunity between this infection and classical typhus. The
serum of animals which had recovered was found by Ricketts and Gomez to
possess protective power, and an antiserum may be obtained from rabbits as
a result of repeated intravenous injections of blood containing the agent

This antiserum when injected during the incubation period is effective in preventing the outbreak of the disease in guinea-pigs inoculated with many times the lethal dose. Active immunity can be developed in these animals by inoculation with fresh mixtures of living virus and antiserum. Effective protection against the disease has been obtained in man by injecting several doses of a carbolised suspension made by grinding up highly infected ticks (Spencer and Parker). This was the first vaccine of the kind to be used. Immunisation must be repeated each year.

In various parts of the world there are typhus-like diseases which are tick-borne and resemble Rocky Mountain Fever more or less closely. Of these, São Paulo typhus appears to be identical, while 'Fièvre boutonneuse' is very closely related. They all correspond with Rocky Mountain Fever as regards the Weil-Felix reaction.

São Paulo Typhus (Brazilian Spotted Fever). Towards the end of 1929 this apparently new disease broke out in the region of Brazil, whose name it bears. It does not spread from man to man, but presumably is derived from wild animals. The clinical features closely resemble those of Rocky Mountain Fever. The tick responsible for its transmission is *Amblyomma cajennense*. The causal rickettsia multiplies in the cornea in guinea-pigs after inoculation into the anterior chamber of the eye. There is complete cross-immunity between Rocky Mountain Fever and São Paulo typhus.

Fièvre boutonneuse, which occurs in Tunis and elsewhere on the shores of the Mediterranean, is spread by a tick, *Rhipicephalus sanguineus*, from dogs, which are frequently infected. The name of the disease indicates the local nodule which develops at the site of the infective tick bite, this is usually on the leg. While guinea-pigs which have recovered from infection with *R. rickettsi* are immune to the rickettsia of *Fièvre boutonneuse* (*R. conori*) and vice versa, a killed vaccine of the former prepared from ticks protects only against itself and not against *R. conori*. Accordingly, the two organisms appear to be variants.

Similar tick-borne typhus fevers occur in India (Megaw), and in South Africa where the condition is designated 'tick-bite fever'. The vectors are not definitely known, and the immunological relationships are not clearly defined. In African tick-borne typhus, patients' sera agglutinate especially the OX-2 strain of *B. proteus*.

RICKETTSIA ORIENTALIS (OF TSUTSUGAMUSHI DISEASE) ETC.

An infection resembling typhus fever, first investigated in Japan, was named "tsutsugamushi disease" because its association with mites was noted. It was also known as "river fever," since it occurs along the banks of large rivers at times of flood. Later, however, it has been recognised in various regions and because of its incidence in country districts has been called rural (or scrub) typhus. It exists in the Malay States and Sumatra, and the disease caused very serious losses among the troops in the campaigns in Burma and New Guinea in the late war. It is transmitted by mites akin to the 'harvest bug', which acquire the infection in one phase of their larval life when they attach themselves to the skin of vertebrate hosts whose lymph they suck. The mites concerned are *Trombicula akamushi* and *T. deliensis*; these attack many species of animals, also birds. Various rodents harbour *R. orientalis* as an inapparent infection, enlargement of the spleen being the only obvious abnormality, thus rats and voles appear to be the natural reservoir. The rickettsia persists in the adult mite and is transmitted to its eggs; so man is infected when attacked by larvæ of the generation following that which

acquired the infection. In certain localities a local lesion of the skin develops at the site of inoculation, but this eschar is not a constant feature of the disease. *R. orientalis* was demonstrated by Ishiware and Ogata, who inoculated rabbits intratesticularly with infective material and found the rickettsia in the interstitial cells of the organ. Nagayo *et al* showed that the infection may be passed in series through rabbits by inoculation into the anterior chamber of the eye; an acute local inflammatory reaction develops with the appearance of the rickettsia in the endothelial cells of Descemet's membrane, this was the first satisfactory method of maintaining the infection. Guinea-pigs are less susceptible than to the organisms of louse-borne and murine typhus or Rocky Mountain Fever, but animals which have been fed on a deficient diet are more readily infected. Monkeys are also susceptible, and develop a local lesion on intradermal inoculation. *R. orientalis* may be accommodated in the mouse and cotton rat (*Sigmodon hispidus hispidus*) on repeated passages by means of intranasal inoculation, a fatal infection resulting (Fulton and Joyner). Pneumonic lesions develop and the lungs contain enormous numbers of the organisms, this material forms the source of suspensions used as vaccines. Growth of the rickettsia occurs in tissue cultures, but not so satisfactorily in the yolk sac of chick embryos. The organism resembles *R. prowazeki* and *R. rickettsi*, but tends to be shorter and plumper and to appear as a bacillus, the average size being 0.8 to 2 μ long and 0.3 to 0.5 μ broad. They appear scattered through the protoplasm of infected cells and also present bipolar forms.

For staining very small rickettsial forms of *R. orientalis* in lung smears Fulton and Joyner proceed as follows. The smears which must be thin and contain as little blood as possible, are fixed by heat and treated with (a) Ziehl-Neelsen's carbol-fuchsin diluted 1 in 6 with distilled water for one to two minutes, washed and differentiated with 50 per cent alcohol till pale pink. (b) then stained for two minutes with a mixture consisting of 50 c.c. saturated solution of thionin in 50 per cent alcohol, 0.5 gm. azure II, 10 c.c. Sørensen's phosphate buffer pH 7.6, distilled water to 100 c.c.

The pathological changes in the organs in man resemble those of typhus fever, but tend to be less pronounced. Recovery from infection is followed by immunity in man and animals, but this is stated to be less durable than in the case of the other typhus-like diseases. The sera of convalescents give the complement-fixation reaction with suspensions of *R. orientalis*. Cross-immunity tests have shown that there is no common immunity to *R. orientalis* and *R. prowazeki*, *R. mooseri*, or *R. rickettsi*. On the other hand, the mite-borne infections of various localities—Japanese, Malayan, Sumatran, etc.—immunise against each other. One source of fallacy which must be considered in such tests is that a strain of low virulence may not immunise successfully against one of higher virulence. Also there is evidence that different strains of this rickettsia may vary in their antigenic components (Rights, Smadel *et al*). By means of a formalised vaccine prepared from infected cotton rats' lungs, mice can be actively immunised against an otherwise fatal infection with the organism. In the second week of the disease as a rule, a positive Weil-Felix reaction develops to the strain of *B. proteus* known as 'Kingsbury' (OX-K) and reaches its height in the third or fourth week, but the reaction is negative with OX-19 and OX-2. Apparently *B. proteus* OX-K and *Spirillum minus* have a common antigen, since rabbits inoculated with *Sp. minus* develop a positive Weil-Felix reaction to this strain (Savoor and Lewthwaite).

RICKETTSIAL POX In 1946 a localised outbreak occurred in New York of a disease characterised by a primary skin lesion followed after about a week by fever and a diffuse eruption which was at first papular, later becoming

vesicular. The cases had a wide age-distribution, but none was seriously ill. Of mice injected intraperitoneally with blood from a patient on the second day of fever several became ill and from one of these the infection was passed to mice and to guinea-pigs, for which it also proved pathogenic, producing a scrotal reaction. Further, on inoculation into the yolk sac of fertile eggs the embryos died and rickettsiæ were demonstrated. The complement-fixation reaction was obtained with the sera of convalescents and an antigen prepared from the yolk sac of infected eggs. A close antigenic relationship was shown to exist between the new rickettsia and *R. rickettsi*, but none with the rickettsiæ of endemic typhus, tsutsugamushi disease or Q fever. The patients' sera did not develop significant agglutinins for *B. proteus* OX-19, OX-2, or OX-K. The same rickettsia was isolated from mites (*Allodermomyssus sanguineus*) and mice in the houses where the outbreak occurred. The serum of the mice also contained antibodies for the rickettsia. Accordingly it is concluded that these animals and the mites which parasitise them are the source of the disease, and the organism has been named *Rickettsia acari* (Huebner *et al.*, see Greenberg *et al.*).

RICKETTSIA BURNETI (*RICKETTSIA DIAPORICA*)

Derrick and Burnet and associates found that guinea-pigs and mice could be infected from the blood of patients who were workers in a slaughter-house and meat-canning factory or on dairy farms in Queensland. The patients suffered from an indeterminate febrile illness without rash and of low fatality, named "Q fever". A rickettsia was demonstrated in the liver and spleen of infected mice. This organism was then shown by cross-immunity tests to correspond to a rickettsia recovered by Davis and Cox from ticks in Montana, U.S.A. and afterwards shown to infect man. The latter was named *R. diaporica* because it passed through filters which retained the causal agents of typhus fever and Rocky Mountain Spotted Fever; *R. burneti* is also filterable. In a fatal case the chief post-mortem change was atypical pneumonia, which suggests that the infection is acquired by inhalation. The serum of infected individuals agglutinates the homologous rickettsia, but not any of the standard *B. proteus* cultures. There is no cross-immunity with *R. rickettsi*. In Queensland a small marsupial, the bandicoot, is infected and several species of ticks are probably vectors, including *Ixodes holocyclus*; this is the likely vector of infection to cattle and farm workers. The organisms multiply in the epithelial cells of the alimentary canal of the ticks and are present in the faeces. Inhalation of this material derived from cattle ticks (*Baophilus annulatus*, *Hæmaphysalis bancrofti*) appears to be the mode of infection of meat-factory workers in the Allied troops in clinically 'atypical pneu

BACTERIOLOGICAL DIAGNOSIS OF TYPHUS FEVERS

This depends practically on the use of the Weil-Felix reaction, since the procedures for recovering the rickettsia by animal-inoculation and its demonstration by staining do not yield results rapidly.

Method. The test should be carried out by the naked eye method in tubes of $\frac{1}{8}$ -in diameter with round bottoms. Standard agglutinable suspensions may be obtained from the Central Public Health Laboratory, London, but instead suspensions may be used in the living state or preserved by alcohol (similar to the standard reagents, see below). For a preliminary test, 1 c.c. of doubling dilutions of the patient's serum

from 1 in 20 to 1 in 640 are measured into the tubes and 0.05 c.c. (one drop) of concentrated bacterial suspension added to each. The mixtures are kept for two hours at 37° C., since O agglutinins of low titre sera tend to be labile at 50° C., the results are read after twenty-two hours further at room-temperature (in the ice-chest in tropical countries). The titre is noted which corresponds with 'standard' agglutination (Appendix). Positive results may be detected by a magnifying lens at the end of the period at 37° C. A positive result with a weakly reacting serum may be missed unless it is heated beforehand at 45° C. for thirty minutes, which eliminates properties inhibiting agglutination.

Preparation of agglutinable suspensions. Cultures must be in the O state. To ensure this they should be plated on agar and discrete colonies selected, if a spreading growth occurs the culture should be plated on medium containing an anti-spreading agent (or 1,500 phenol may be used), then a number of colonies are subcultured separately on ordinary agar and non-spreaders chosen. After incubation for twenty-four hours at 37° C. those are selected which form stable suspensions in 3.4 and 6.8 per cent NaCl solution, i.e. are not in the rough state (this does not apply to the OX-K strain). The cultures have then to be examined for agglutinability with a known positive serum or, less satisfactorily, with rabbit's antiserum. Two agar slope cultures are made from each colony and grown at 37° C. for twenty-four hours. One of these is kept ('duplicate'), the other is suspended in 2 c.c. saline poured into a fresh test-tube and at least four volumes of 96 per cent alcohol added. The tubes are shaken repeatedly in the course of one hour, then the suspension is centrifuged, the sediment resuspended in 0.25 per cent formal-saline and diluted to a suitable density. That culture is finally selected the alcohol-treated suspension of which is agglutinated most rapidly and completely to the highest titre. Bulk suspensions may then be prepared from twenty-four hours' growths on agar in Roux flasks or screw-capped 'medical flat' bottles—these are inoculated with a broth culture made from the 'duplicate'. After treatment of the centrifuged sediment with alcohol, as described above, as much as possible of the supernatant fluid must be pipetted off, the sediment is then thoroughly resuspended in sterile saline and to this is added 1/7th of its volume of 2 per cent formal-saline which has been brought to a pH of 7.6 by addition of Na_2HPO_4 , thus the final concentration of formal is 0.25 per cent in the mixture. The density of this suspension is standardised as follows: ascertain how many times it must be diluted in order to match No. 3 of Brown's opacity tubes, say twelve times, the required density is usually 3.7 times this. Accordingly, 50 c.c. of suspension will require to be diluted to 182 c.c., i.e. 112 c.c. of diluent must be added, of which 7/8ths is sterile saline (98 c.c.) and the remaining 1/8th (14 c.c.) is 2 per cent formal-saline.

In the case of *B. proteus* OX-K, the suspension after treatment with alcohol should be diluted with sterile distilled water and then the required amount of 2 per cent formal-saline added. Sensitive stable suspensions are not readily obtained with this organism.

Interpretation of results. According to Felix, in louse-borne typhus complete agglutination of the standard suspension of *B. proteus* OX-19 with serum diluted 1 in 80 is significant for a patient, not a native of an endemic area, who has not been recently vaccinated with typhus vaccine. Where there has been anti-typhus vaccination within three months complete agglutination with a dilution of 1 in 200 or over strongly suggests active infection. A marked increase in titre, i.e. doubling, in tests repeated at two-day intervals is positive evidence. Failure of the titre to rise practically excludes louse-borne typhus except in very severe cases likely to prove fatal. A drop from a high titre during a period covering three to four months after the illness is also to be accepted as evidence that a clinically mild condition was typhus fever. A later febrile illness of another nature does not cause rise of the agglutinins for OX-19. About 25 per cent of cases never show a titre as high as 1 in 500, in these a positive reaction is obtained later (sixth to seventh day) than in strongly reacting cases. Mild illnesses may show either a high or low titre. In murine typhus the reaction appears to have the same diagnostic value as in the louse-borne disease.

In rural typhus the use of the reaction with the OX-K strain is qualified by the fact that suspensions of this organism are less stable than those of the other *B. proteus* strains. Accordingly, a titre not under 1 in 160 for total agglutination is required for a significant result on a single examination. As

ferments or dyes, may be retained by a filter. Thus the retention of a supposed virus by a filter does not necessarily mean that it is too large to pass through the pores. It has been estimated that the pores of the Berkefeld filters are larger than some of the bacteria which are retained by such filters. Accordingly the passage of a particle through a pore

In recent times organic membranes such as collagen have been employed to test filterability. This work has been developed by Elford and a method has been evolved for obtaining membranes of uniformly graded porosity—*gradocol* membranes. The average diameter of the pores of these membranes ranges from $3\ \mu$ to $10\ m\mu$ ($0.01\ \mu$). They are prepared by evaporating a solution of collodion in acetone to which has been added a mixture of ethyl alcohol and ether along with varying quantities of amyl alcohol. The size of the pores is determined by the composition of the collodion solution and the conditions of evaporation. By means of such membranes Elford and his co-workers have computed the sizes of the particles representing various filterable viruses: these vary from 8 to $12\ m\mu$ (*pohomyelitis* virus) to 0.125 to $0.175\ \mu$ (*vaccinia* virus). This range of size is striking when compared with the much narrower limits of the ordinary bacteria.

The filterable viruses have also been described as ultramicroscopic, though in certain virus diseases, e.g. *vaccinia*, very minute coccoid bodies—'elementary' bodies—can be recognised by suitable microscopic methods; they were originally described by Buist in 1886. There are good grounds for considering that these are the actual virus. Elementary bodies are often found in pairs or short chains, suggesting that they are in process of division. It has been generally assumed that micro-organisms or particles of less diameter than $0.2\ \mu$ are below the limit of the resolving power of the microscope and so cannot be distinguished as separate objects by ordinary microscopic methods. Coles, however, has pointed out that with white light as the illuminant and an objective of N.A. 1.4 a dark isolated particle just over $0.074\ \mu$ in diameter is visible, and with green light an even smaller particle ($0.0673\ \mu$ diameter) can be seen. It has been estimated that the Berkefeld V filter (one of the most porous varieties) will allow the passage of particles measuring approximately $0.2\ \mu$, so that theoretically an organism might pass through certain grades of filters and yet be demonstrable microscopically. It should be noted that staining methods which involve the use of mordants, etc., probably render virus particles visible by incrusting them with a deposit of stain, thus exaggerating their real size. For the study of virus particles, Barnard has utilised photography by ultra-violet light, which is of shorter wavelength than that of the visible rays, and thus has obtained greater microscopic resolution. (For this purpose quartz lenses are required, as glass absorbs the ultra-violet rays.) By this method he has been able to demonstrate photographically virus particles of $0.075\ \mu$ in diameter. Dark-ground illumination, which renders visible very minute bodies not readily demonstrable with the ordinary microscope, has not contributed much to our knowledge of the ultra-microscopic viruses, owing to the fact that an organism of such minute dimensions, unless actively motile, cannot be distinguished from the numerous particles seen by this method in all organic material. The recent introduction of the electron microscope with its great resolving power has enabled virus particles to be studied at high magnifications. Thus examination by the electron microscope indicates that the elementary bodies of *vaccinia* virus are brick-shaped and suggests that they possess an internal structure. Many other virus particles are rounded or filamentous.

A method has been developed for estimating the size of virus particles by subjecting them to ultracentrifugal action in an ultracentrifuge (see Wickstead for a description of the Sedley type) which is applicable (Sedley, 1935, p. 113, 14). In this way estimates have been made of the sizes of vaccinia and certain other viruses, which are in close agreement with those determined by filtration through cellulosic membranes.

The same centrifugal method has been used to indicate that viruses are more massive than most bacteria. Thus while some viruses, such as that of vaccinia, have been reported to have a diameter of 0.15 to 0.175 μ , which approaches the dimensions of the smallest bacteria, on the other hand certain viruses have been estimated to be at least 50 times heavier than the protein of a complex protein. It is probably not too far from the truth to say that the size of a virus particle is difficult to estimate. Between the extremes of weight there is usually an enormous variation, and it is impossible in the present state of knowledge to set any definite lower limit to the lower limit of mass. It would be erroneous, of course, to suppose that the range in size of the members of any of the listed groups is that of the ordinary bacteria. While the terms 'filterable' and 'ultrafilterable' have been commonly used, it is apparent that they are not strictly applicable in the description of the viruses as a whole, and there is a growing tendency to refer to this group of minute infective agents as 'viruses' or 'ultraviruses'. One must be taken not to over-estimate the accuracy of physical grounds that small particles recovered from tissues by physical methods are of virus nature, or to conclude that objects may have the virus range of sizes (see Sedley, loc. cit.).

Cultivation (p. 528). An essential property of the filterable viruses is their capacity to propagate in the appropriate conditions, e.g. in the tissues. Attempts have been made to cultivate various viruses artificially and to reproduce their pathogenic effects by means of such cultures. While they have not been cultivated in ordinary artificial media, many of them multiply in life when in association with living tissue cells, e.g. in tissue cultures or, also, with outgrown tissues from animals or in the chorioallantoic membrane of the developing egg (Wickstead and Goodpasture, 1935, 1936, and Burrell). In the latter some viruses produce chiefly focal lesions, 'plaques', in the membrane, e.g. vaccinia, herpes simplex, footpox; others rapidly kill the embryo without causing focal lesions of the membrane, e.g. equine encephalomyelitis; some produce both, e.g. influenza, pertussis, while others may multiply without causing pathological effects, e.g. rabies, measles. A medium of mixed tissue, in which there is very little cellular proliferation, may serve for the cultivation of a virus, e.g. that of vaccinia (Mantidze and Laima). Here the cells may have a two-fold function: (1) to initiate growth of the virus, and (2) to provide conditions which allow the continuation of growth. It is clear that these two functions need not necessarily depend on the same mechanism.

Instability. In general the viruses are susceptible to the same physical and chemical agents as the bacteria. Thus they are relatively sensitive to heat, ultra-violet rays, and antiseptics, also to atmospheric oxygen, though considerable variations do meet with. A remarkable feature of most filterable viruses is their resistance to glycerol, especially at low temperatures. While spore-free bacteria generally are killed by 50 per cent glycerol, the viruses of smallpox, rabies, poliomyelitis, herpes, etc., remain undisturbed for long periods in this fluid at 4°C. This property has been utilised practically in the preservation of the vaccinia virus in calf lymph for smallpox vaccination (*vide p. 567*). Thorough and rapid drying *in vacuo* at a low temperature and storage in the cold appears to be the best method of preserving viruses.

Pathogenic action. The diseases of man and animals due to the filterable viruses are characterised generally by their high degree of infectivity and rapidity of spread. Very minute doses of the virus also are capable of producing a manifest infection. This has been well illustrated under experimental conditions. Thus it has been shown that 0·000001 c.c. of an emulsion of brain tissue from a monkey infected with yellow fever will cause the disease on injection into another animal.

The characteristic pathogenic effects which may follow infection by

viruses and comprise all the known degenerative and reactive tissue changes, as well as the production of simple and malignant neoplasms in certain cases. Many of the symptoms of virus infection suggest the action of toxins, but the production of these bodies has seldom been demonstrated. Viruses, like bacteria, are subject to adaptation, e.g. exaltation and attenuation of virulence, so that the severity of the disease effects produced by a given virus may vary within wide limits. Apparently spontaneous variation (mutation) may lead to similar results. Also, latent infections are frequently met with, and likewise carriers.

The method of passage carried on with a view to raising the virulence of a virus agent suspected to be present, has brought to light a source of fallacy in such investigations. Apparently normal animals may harbour a virus which only becomes virulent after repeated passages. Thus 'virus III' of rabbits (Rivers and Tillett) was discovered as the result of repeated transfers of testicular material through a series of animals, the original inoculation having been with blood of rabbits which seemed healthy (Andrewes and Miller). In the first passages no changes followed the injections, but in later transfers the intratesticular inoculation produced acute orchitis and pyrexia; and intranuclear cell inclusions were found, especially in the macrophages. Other species of animals were not susceptible. Similarly the salivary glands of guinea-pigs appear to contain frequently a transmissible virus which causes a local mononuclear reaction and the formation of intranuclear inclusion bodies. Intratesticular inoculation of the virus causes similar lesions, and injection into the brain leads to acute meningitis. Other examples will be mentioned later.

In the majority of virus infections special intracellular structures have been found—*inclusion bodies*. These are in some diseases restricted to a particular tissue, but in others they are present in the cells of various tissues. In certain diseases the bodies occur in the cytoplasm, in others they are intranuclear, and they may be met with in both situations. They vary greatly in size from about 0·2 μ upward, and in the cytoplasm of a single cell there may be forms measuring 1 to 20 μ or more. The cytoplasmic inclusion bodies tend to stain with acid dyes, but often they contain also basophile constituents. The intranuclear bodies are eosinophile. For their demonstration Giemsa's solution or other eosin-methylene-blue combination, or Mann's methyl-blue-eosin stain have been much used. They usually fail to give the Feulgen reaction (the psittacosis and allied viruses, and Guarnieri bodies excepted—Lépine and Sautter) and do not contain masked iron, also the oxidase reaction is negative, while on micro-incineration they yield no ash (in contrast to nucleoli), but certain inclusions, e.g. Negri bodies, contain calcium. Various views have been held as to the nature of these inclusion bodies, it is probable that they represent the virus particles or aggregates of these surrounded by cellular products, either reactive or degenerative, and derived from both nucleus and cytoplasm. In accordance

with the conception that inclusion bodies represent parasites 'cloaked' with cellular material they were at one time called 'chlamydozoa'. In the case of the Bollinger bodies in fowlpox, the disease has been reproduced by inoculation with one such body, whereas the fluid in which it had been washed proved non-infective (Woodruff and Goodpasture). In some cases by suitable methods the bodies can be shown to be composed of minute granules, *elementary bodies*, and these correspond to the minute bodies which may be observed in lesions as free structures, *e.g.* in the pox diseases. In certain cases, *e.g.* the Negri and Guarmeri bodies of rabies and vaccinia

be demonstrated. Cell inclusions and elementary bodies have been seen to develop *in vitro* in tissue cultures containing a virus. These facts suggest that the filterable viruses are intracellular parasites, and in this respect they contrast with most of the ordinary bacteria. While inclusion bodies are highly characteristic of the action of viruses, it cannot be stated that these are their sole cause. It has been claimed that they can be produced by various physical and chemical agents (see Fischmann and Russell).

Certain virus diseases are practically confined to man, among those generally accepted are smallpox, epidemic poliomyelitis, encephalitis lethargica and several other forms of encephalitis, herpes simplex (febrilis), herpes zoster, chicken-pox, measles, the common wart, molluscum contagiosum, trachoma, inclusion conjunctivitis, epidemic kerato-conjunctivitis, climatic bubo (lymphogranuloma inguinale), dengue, phlebotomus fever and other three-day fevers of tropical and subtropical countries, mumps, influenza, the common cold, infective hepatitis, and glandular fever. In all, over thirty human diseases have been ascribed to viruses. Others which are common to man and certain animals are vaccinia, rabies, yellow fever, Rift Valley Fever, psittacosis, and several other forms of 'atypical' pneumonia, lymphocytic choriomeningitis, and louping-ill, also various forms of encephalomyelitis.

In addition, a number of prevalent animal diseases are due to similar viruses—foot-and-mouth disease, the pox diseases of various animals and birds, distemper of dogs, swine-fever (hog cholera), cattle plague (rinderpest), African horse sickness, fowl-pest, vesicular stomatitis of horses, Borna disease of horses, infectious anaemia of horses, pseudo-rabies of cattle, leukaemia of fowls, ectromelia of mice, and various others. Swine influenza is an example of a 'complex infection', where the combined action of a virus with a bacterium (*B. influenzae suis*) produces a severe disease, whereas each by itself has little or no pathogenic effect (Shope).

While in most of the above diseases the evidence that the specific etiological factor is a filterable virus has been well established, in some cases it is not altogether conclusive or complete. These questions will be discussed later.

A filterable causal agent was also demonstrated by Rous in 1911 in cases of fowl sarcoma, and has given rise to much speculation and discussion regarding the possible virus etiology of malignant tumours. Though certain lesions which might be classified as simple neoplasms (*vide supra*) have been found to be due to viruses in mammals, the only causal virus like that of the Rous sarcoma which has been discovered in a malignant tumour of animals other than certain birds is associated with myxomatosis of rabbits—although this may be a complex infection with two viruses (Shope).

Levaditi pointed out that a group of filterable viruses, *e.g.* those of

Pathogenic action. The diseases of man and animals due to the filterable viruses are characterised generally by their high degree of infectivity and rapidity of spread. Very minute doses of the virus also are capable of producing a manifest infection. This has been well illustrated under experimental conditions. Thus it has been shown that 0·000001 c.c. of an emulsion of brain tissue from a monkey infected with yellow fever will cause the disease on injection into another animal.

The characteristic pathogenic effects which may follow infection by a virus and its multiplication in the tissues constitute one of the chief means of recognising its presence. These effects in general are similar to those produced by bacteria and comprise all the known degenerative and reactive tissue changes, as well as the production of simple and malignant neoplasms in certain cases. Many of the symptoms of virus infection suggest the action of toxins, but the production of these bodies has seldom been demonstrated. Viruses, like bacteria, vary in their degree of virulence, so that the virus may vary with (mutation) may be met with, and likewise carriers.

The method of passage carried on with a view to raising the virulence of a virus agent suspected to be present, has brought to light a source of fallacy in such investigations. Apparently normal animals may harbour a virus which only becomes virulent after repeated passages. Thus 'virus III' of rabbits (Rivers and Tillett) was discovered as the result of repeated transfers of testicular material through a series of animals, the original inoculation having been with blood of rabbits which seemed healthy (Andrewes and Miller). In the first passages no changes followed the injections, but in later transfers the intratesticular inoculation produced acute orchitis and pyrexia; and intranuclear cell inclusions were found, especially in the macrophages. Other species of animals were not susceptible. Similarly the salivary glands of guinea-pigs appear to contain frequently a transmissible virus which causes a local mononuclear reaction and the formation of intranuclear inclusion bodies. Intratesticular inoculation of the virus causes similar lesions, and injection into the brain leads to acute meningitis. Other examples will be mentioned later.

In the majority of virus infections special intracellular structures have been found—*inclusion bodies*. These are in some diseases restricted to a particular tissue, but in others they are present in the cells of various tissues. In certain diseases the bodies occur in the cytoplasm, in others they are intranuclear, and they may be met with in both situations. They vary greatly in size from about 0·2 μ upward, and in the cytoplasm of a single cell there may be forms measuring 1 to 20 μ or more. The cytoplasmic inclusion bodies tend to stain with acid dyes, but often they contain also basophile constituents. The intranuclear bodies are eosinophilic. For their demonstration Giemsa's solution or other eosin-methylene-blue combination, or Mann's methyl-blue-eosin stain have been much used. They usually fail to give the Feulgen reaction (the psittacosis and allied viruses, and Guarnieri bodies excepted—Lépine and Sautter) and do not contain masked iron, also the oxidase reaction is negative, while on micro incineration they yield no ash (in contrast to nucleoli), but certain inclusions, e.g. Negri bodies, contain calcium. Various views have been held as to the nature of these inclusion bodies; it is probable that they represent the virus particles or aggregates of these surrounded by cellular products, either reactive or degenerative, and derived from both nucleus and cytoplasm. In accordance

with the conception that inclusion bodies represent parasites 'cloaked' with cellular material they were at one time called 'chlamydozoa'. In the case of the Bollinger bodies in fowlpox, the disease has been reproduced by inoculation with one such body, whereas the fluid in which it had been washed proved non-infective (Woodruff and Goodpasture). In some cases by suitable methods the bodies can be shown to be composed of minute granules, *elementary bodies*, and these correspond to the minute bodies which may be observed in lesions as free structures, *e.g.* in the pox diseases. In certain cases, *e.g.* the Negri and Guarnieri bodies of rabies and vaccinia respectively, the cell inclusions are specific in the sense that their morphology and distribution are highly characteristic for the particular disease. But, of course, the virus may be present in material in which inclusion bodies cannot be demonstrated. Cell inclusions and elementary bodies have been seen to develop *in vitro* in tissue cultures containing a virus. These facts suggest that the filterable viruses are intracellular parasites, and in this respect they contrast with most of the ordinary bacteria. While inclusion bodies are highly characteristic of the action of viruses, it cannot be stated that these are their sole cause. It has been claimed that they can be produced by various physical and chemical agents (see Fischmann and Russell).

Certain virus diseases are practically confined to man; among those generally accepted are smallpox, epidemic poliomyelitis, encephalitis lethargica and several other forms of encephalitis, herpes simplex (febrilis), herpes zoster, chicken-pox, measles, the common wart, molluscum contagiosum, trachoma, inclusion conjunctivitis, epidemic kerato-conjunctivitis, climatic bubo (*lymphogranuloma inguinale*), dengue, phlebotomus fever and other three-day fevers of tropical and subtropical countries, mumps, influenza, the common cold, infective hepatitis, and glandular fever. In all, over thirty human diseases have been ascribed to viruses. Others which are common to man and certain animals are vaccinia, rabies, yellow fever, Rift Valley Fever, psittacosis, and several other forms of 'atypical' pneumonia, lymphocytic choriomeningitis, and louping-ill, also various forms of encephalomyelitis.

In addition, a number of prevalent animal diseases are due to similar viruses—foot-and-mouth disease, the pox diseases of various animals and birds, distemper of dogs, swine-fever (hog cholera), cattle plague (rinderpest), African horse sickness, fowl-pest, vesicular stomatitis of horses, Borna disease of horses, infectious anaemia of horses, pseudo-rabies of cattle, leukaemia of fowls, ectromelia of mice, and various others. Swine influenza is an example of a 'complex infection', where the combined action of a virus with a bacterium (*B. influenzae suis*) produces a severe disease, whereas each by itself has little or no pathogenic effect (Shope).

While in most of the above diseases the evidence that the specific etiological factor is a filterable virus has been well established, in some cases it is not altogether conclusive or complete. These questions will be discussed later.

A filterable causal agent was also demonstrated by Rous in 1911 in cases of fowl sarcoma, and has given rise to much speculation and discussion regarding the possible virus etiology of malignant tumours. Though certain lesions which might be classified as simple neoplasms (*vide supra*) have been found to be due to viruses in mammals, the only causal virus like that of the Rous sarcoma which has been discovered in a malignant tumour of animals other than certain birds is associated with myxomatosis of rabbits—although this may be a complex infection with two viruses (Shope).

Levaditi pointed out that a group of filter-passing viruses, *e.g.* those of

rabies, poliomyelitis, encephalitis, vaccinia, and herpes, resemble one another not only in their general characters, but also in possessing in common an affinity for ectodermal structures—the nervous system on the one hand and the skin, cornea, etc., on the other. He applied the general term *ectodermoses* to the resulting lesions and regarded namely, *neurotropic* and *dermotropic*. myelitis virus is classed as the most

extreme of the series is the vaccinia virus, being specially dermatropic; the virus of herpes occupies an intermediate position, producing lesions both in the skin and central nervous system in experimental animals. It may be mentioned in this connection that, as shown by Marie, the vaccinia virus can be adapted to growth in the cerebral tissue; thereafter this 'neuro-vaccine' can be maintained indefinitely in series, and it then possesses a fixed virulence, as in the case of the virus of rabies. The work of Ledingham especially has shown, however, that cells of epiblastic origin are not those chiefly affected by vaccinia virus, since the lesion is essentially an infective granuloma in which the reticulo-endothelial system is primarily and dominantly involved.

As regards the pathological effects produced by neurotropic viruses, it must be emphasised that certain filterable viruses under natural conditions produce diseases in man or animals in which the lesions affect chiefly or exclusively the central nervous system. Also susceptible animals when infected with these experimentally by inoculation at sites remote from the brain and spinal cord, as a rule develop similar lesions. Some other viruses, again, show little or no tendency to localise in the central nervous system, but when brought into direct contact, by intradural or intracerebral inoculation, they exert pathogenic action locally. To all such viruses the term 'neurotropic' has been loosely applied. Hurst has suggested a more satisfactory pathological basis for their classification as follows. (a) The *strict neurotropes*, such as poliomyelitis, rabies, pseudo-rabies (in the monkey), and Borna disease viruses. These attack directly and destroy nerve cells, other lesions being secondary. They reach the central nervous system from a peripheral site of inoculation by the nerves of the part. They multiply only in nervous tissue and they do not appear in the blood or spinal fluid till the disease is far advanced. Intravenous inoculation usually does not succeed except with large doses. (b) The *panotropic viruses* one type, such as those of herpes febrilis (in the rabbit) and pseudo-rabies (in the rabbit), though neurotropic also attacks cells of all the embryonic layers. Neural spread occurs. A second type, represented by yellow fever and louping-ill, is more restricted in its affinities, and the nervous lesions tend to develop late in the disease, after antibodies have appeared in the blood. The latter type is often spread by blood-sucking arthropods.

doses often succeeds circulation. (c) The *viscerotropic viruses*, such as those of lymphogranuloma inguinale, when inoculated directly into the brain produce primarily meningeal lesions and any damage of nerve cells is secondary. Under natural conditions they do not produce encephalitis.

Immunity to virus diseases exhibits the same wide range of duration, e.g. smallpox, measles, poliomyelitis, etc., and this result may also follow inoculation with the virus in an attenuated form, e.g. the vaccinia virus, whereas after others susceptibility to reinfection soon returns, e.g. herpes, influenza, etc. Certain of the viruses when in the 'dead' or 'inactive', i.e. non-infective, state also act as effective vaccines. Thus

viruses are antigenic. But the development of antibodies in the blood does not necessarily indicate active immunity. The serum of recovered or immunised animals may deprive the virus of infectivity, as proved by failure of a susceptible animal to develop lesions on inoculation with a mixture of the two. The exact mechanism of the anti-infective action of the immune serum is not clear, however. It may sensitise the virus to the destructive action of the host's cells and body fluids or it may in some way 'neutralise' the virus. The varying results which may be obtained according to the route by which a mixture of immune serum with virus is introduced into the body suggests that the mechanism of immunity is intimately related to the particular cells to be protected (see Sabin). Passive immunity may be conferred by injections of the immune serum. Sometimes fixation of the antibody by the virus *in vitro* can be shown to occur, but there may be a protective effect although it can be proved that the virus is not killed *in vitro* by the antiserum even in the presence of complement. Flocculation and complement-fixation reactions resulting from the interaction of virus and antibody have also been demonstrated *in vitro*, although the results have frequently been negative. In investigating the identity of viruses from different sources, extensive use has been made of the specific anti-infective action of immune sera or their flocculating property, viruses whose infectivity is neutralised by the same antiserum or which are flocculated by it in similar degree, are considered to be homologous. Where concentrated and purified homogeneous suspensions of the elementary bodies from the lesions have been obtained, as in the case of vaccinia and fowlpox, it has been shown that they are agglutinated specifically by the sera of animals which have recovered from the respective infections or have been immunised with the corresponding viruses, normal sera lack this agglutinating action. Such serological evidence supports strongly the view that these elementary bodies constitute the actual infective agent. Some viruses, *e.g.* that of vaccinia, contain multiple antigens. Different immunological types may also be recognised, *e.g.* in the virus of foot-and-mouth disease.

Interference effects. It has been observed that the pathogenic action of a virus may be annulled by inoculation along with either the same virus (at a different site) or a modification of it or an unrelated virus. Thus a susceptible monkey injected subcutaneously or intraperitoneally with virulent pantropic yellow fever virus will survive provided it receives simultaneously or after an interval of less than twenty-four hours an injection of the neurotropic (*tc* modified) strain by similar route (Hoskins). Similarly, a herpes virus which produces fatal encephalitis in the rabbit when inoculated either on the scarified cornea or into the brain, causes no ill effect provided that the corneal inoculation is followed by an intracerebral injection of the virus at an appropriate time during the incubation period (Magrassi, Doerr *et al.*). Findlay and MacCallum have shown, too, that Rift Valley Fever virus, which is immunologically unrelated to pantropic yellow fever virus, protects against the lethal action of the latter when a mixture is injected subcutaneously or intraperitoneally into susceptible monkeys. Immunity reactions appear to play no part in the phenomenon. Interference has also been observed with a mixture of different influenza viruses in the developing chick embryo. A virus rendered non-infective by ultra-violet radiation, is likewise capable of producing this interference. Accordingly, it would seem that in order to effect interference the two viruses must have affinities for the same constituents of the host's cells (see Ziegler and Horsfall). A complete explanation of these interference effects cannot yet be given. But they may account, in part at least, for the prophylactic action of Pasteur's rabies

vaccine. Interference may also be a contributory factor in Webster's observation that a subcutaneous injection of the virus of St. Louis encephalitis into mice protects against an otherwise fatal infection.

and Webster). The highest dose of virus has ever reached the brain (Hodes

Methods of identification. Means are generally lacking for isolating viruses and demonstrating distinctive characters in cultures. Accordingly, in order to identify a virus with one of the known species, it is necessary to rely largely on the following points. (a) The species of animals susceptible to infection and the resulting symptoms and lesions, also the sites from which the virus may be recovered (e.g. blood, various organs, cerebro-spinal fluid, etc.). (b) The natural modes of transmission (droplet infection, insects, etc.), and the routes by which experimental infection can be produced. (c) Is there complete cross immunity between a known virus and that under investigation? Also does an antiserum to the virus in question react quantitatively with it in the same way as the antiserum to a known virus and vice versa? If all these features and methods of investigation show that two viruses are identical, then they are identical. On the other hand, if different it is necessary to consider

due to adaptations or variations such as can also be demonstrated experimentally in the known virus.

Nature of viruses. It is at present impossible to make comprehensive statements regarding the nature of viruses owing to their heterogeneous characters. Certain salient features, however, will be mentioned. Viruses originally attracted attention because of their pathogenic properties and it was found that the particular disease could be reproduced indefinitely by passage through a series of hosts. It is beyond doubt that they increase in the tissues. Accordingly, they possess a capacity of reproduction which is the chief attribute of living agents. In addition, they exhibit adaptation or variation, e.g. alterations in virulence; and they possess an individual specificity in the serological sense. But further analysis of their characters raises the question of what is meant by life, e.g. in the case of the smaller viruses which appear to have a mass of not more than several protein molecules; also, some plant virus aggregates are rods or 'crystals' of very high molecular weight (Stanley); animal viruses have been proved to be of

it is difficult to make such a homogeneous entity fit in with current conceptions of life, which involve a living object being an organised structure. On the other hand, the larger viruses are but little smaller than the ordinary bacteria, and in addition to protein they have lipid and carbohydrate constituents, so that from the point of view of organisation they may be accepted as akin to the bacteria. But no virus is able to proliferate in the absence of living cells, this is a feature which they share with bacteriophages ('bacterial viruses'). Further, no definite evidence exists of their possessing metabolic functions. Hence it has been concluded that they represent an extreme degree of parasitism, being practically entirely dependent for their growth on the products of the living cells with which they are associated. Since, in general, the evidence indicates that parasites have evolved from free living forms, it is concluded that viruses must be derived from ordinary bacteria. This view would exclude the possibility, entertained by some, that

viruses can develop by spontaneous generation, a living thing being derived from something unlike itself (heterogenesis), or, to use a chemical expression, that viruses are the products of an autocatalytic process whereby the presence of the agent causes a substrate to be transformed into it (A process of this kind is exemplified by the part which certain zymases play in changing the corresponding zymogen into the zymase.) However, this analogy does not take into account the way in which the original moiety of the virus could arise spontaneously in the individual host. Certainly there is no epidemiological evidence which requires the assumption that any virus develops *de novo*. The conception that viruses are microbic genes also lacks an experimental basis so far

RABIES OR HYDROPHOBIA

Rabies or hydrophobia is an infective disease which in nature occurs epizootically chiefly among the carnivora, especially in the dog, wolf, jackal, and cat. Infection is transmitted by the bite of a rabid animal, or by a wound or abrasion being licked by such an animal. The disease can be transferred to other species, and when once started can be spread from individual to individual by the same paths of infection. Thus it occurs from time to time in cattle, sheep, pigs, horses, and deer, and can be communicated to man. Cases of infection from man to man by bites are recorded, but the saliva in man does not appear to be so infective as in dogs. It is to be noted that the virus is extremely potent, as cases are on record of infection taking place through an unabraded mucous membrane by the licking of a rabid animal, and the experimental application of the virus to such surfaces as the *mucous membrane of the nose or the conjunctiva is often followed by infection*

In Western Europe the disease is most frequently observed in the dog, but in Eastern Europe, especially in Russia, epidemics among wolves have been a serious danger

common. After a period of incubation of from three to six weeks the first symptom noticed is a change in the animal's aspect, it becomes restless, it snaps at anything which it touches, and tears up and swallows unwonted objects, it has a peculiar high-toned bark. Spasms of the throat muscles appear, especially in swallowing, and there is abundant secretion of saliva. The supposed special fear of water is due to difficulty in swallowing. Gradually convulsions, paralysis, and coma come on, and death supervenes usually about five days after the appearance of symptoms. In the paralytic form, the early symptoms are the same, but paralysis appears sooner. The lower jaw of the animal drops, from implication of the elevator muscles, all the muscles of the body become more or less weakened, and death ensues without any very marked irritative symptoms.

In man the incubation period after infection varies from fifteen days to seven or eight months, or even longer, but is usually about forty days. When symptoms of rabies are about to appear, certain prodromata, such as pains in the wound and along the nerves of the limb in which the wound has been received, may occur. This is succeeded by a stage of nervous irritability during which all the reflexes are exaggerated. There are spasms, especially of the muscles of deglutition and respiration, and cortical irritation indicated by delirium may occur. On this follows a period in which all the reflexes are diminished, weakness and paralysis are observed, convulsions occur, and finally coma and death supervene. The duration of the acute illness is usually from four to eight days, and death invariably results. Rabies in man

sometimes assumes a paralytic form. This is usually manifested at first by paralysis of the limb in which the infection has been received, and of the neighbouring parts, and then the occurrence of widespread and progressive paralysis is the outstanding feature. In man there occur cases where the cerebellum and also the sympathetic system seem to be specially affected. Among persons bitten by rabid dogs the incidence is comparatively low (*vide infra*); the death-rate after the bites of wolves is 80 per cent. Here, however, it must be kept in view that, as the wolf is naturally the more savage animal, the number and extent of the bites, i.e. the number of channels of entrance of the infection into the body and the total dose are greater than in the case of persons bitten by dogs. A peculiar outbreak of rabies in Trinidad, taking the form of an acute ascending myelitis, was studied by Hirst and Pawan. Their observations indicated that the infection originated from cattle, in which the disease was present at the same time, and was transmitted by blood-sucking vampire bats. A similar type of rabies infection in animals has been reported from Brazil.

Pathological Effects and Experimental Inoculation. In rabies the appearances discoverable by an ordinary examination of the nervous system, to which all symptoms can be referred, are comparatively unimportant. On naked-eye examination, congestion, and, it may be, minute hæmorrhages, especially in the medulla, are the only features noticeable. Microscopically, lymphocytic exudation into the perivascular lymphatic spaces in the nerve centres has been observed, and in the nerve cells various degenerations have been described. Round the nerve cells in the grey matter of the medulla and cord, Babès described accumulations of newly formed cells, and Van Gehuchten observed a phagocytosis of the nerve cells in the posterior root ganglia and also in the sympathetic ganglia. A most important feature, however, is the presence in the nerve cells of the structures known as Negri bodies and these will be discussed later. In the white matter, especially in the posterior columns, swelling of the axis cylinders and breaking up of the myelin sheaths have been noted, and similar changes may occur also in the spinal nerves, especially of the part of the body through which infection has come. The changes in the other parts of the body are unimportant. Apart from the presence of Negri bodies, the pathological changes are similar to those in encephalitis due to various other viruses.

Pasteur's first contribution to the pathology of rabies was to show that the most certain method of inoculation of an experimental animal was by inserting infective matter beneath the dura mater. He found that in the case of any animal or man dead of the disease, injection by this method of emulsions of any part of the central nervous system of the cerebro-spinal fluid, or of the saliva, invariably gave rise to the disease, and the period of incubation was shortened.

lytic forms was proved, as sometimes the one, sometimes the other, was produced, no matter which form had been present in the original case. Inoculation into the anterior chamber of the eye is nearly as efficacious as subdural infection, and so also is injection into a peripheral nerve, e.g. the sciatic. Intramuscular injection into the muscles of the neck leads to infection in a high proportion of cases. Subcutaneous or intravenous injection may or may not give rise to the disease; while intraperitoneal inoculation is very uncertain.

From such inoculation experiments much information has been acquired regarding the spread and distribution of the virus in the body. Gaining entrance by the infected wound, it early manifests its affinity for the nervous tissues. It reaches the central nervous system chiefly by spreading up the

peripheral nerves. This can be shown by inoculating an animal subcutaneously in one of its limbs with virulent material. If now the animal be killed before symptoms have manifested themselves, rabies can be produced by subdural inoculation from the nerves of the limb which was injected. Further, rabies can often be produced from such a case by subdural inoculation with the part of the spinal cord into which these nerves pass, while the other parts of the animal's nervous system do not give rise to the disease. This explains how the initial symptoms of the disease (pain along nerves, paralysis, etc.) so often appear in the affected part of the body, and it probably also explains the fact that bites in such richly nervous parts as the face and head are much more likely to be followed by rabies than bites in other parts of the body. The virus seems to have a selective affinity for the salivary glands, as well as for the nervous system. Roux and Nocard found that the saliva of the dog became infective three days before the first appearance of symptoms of the disease. The blood is usually non-infective, as are also the internal organs except certain glands, e.g. the pancreas.

While rabbits and guinea-pigs have been extensively used for inoculation tests with the rabies virus, it has been shown that mice are more susceptible to intracerebral inoculation, dying more quickly, also such inoculation is more easily carried out in mice (Webster). Certain strains of mice are particularly suitable for inoculation tests.

Rabies Virus. The causal agent of rabies can pass through the coarser Berkefeld filters and also sometimes through the coarser Chamberland filters. This is shown by the fact that if an emulsion of any infective material (e.g. the brain) be thus filtered, the filtrate is also infective. Evidence that it is the virus itself which passes through is found in the fact that when an animal dies after injection with the filtrate, a small portion of its central nervous system will originate the disease in a fresh animal. The infective agent is thus placed among the filterable viruses. Its size has been estimated at 100 to 150 $m\mu$ (Galloway and Elford). Cultivation in the developing egg has been reported (Khgler *et al.*), but this has usually been unsuccessful. Cultures have been obtained in tissue-cultures of embryo brain with human or monkey serum. The resistance of the virus to external agents varies. Thus tissue of the nervous system containing it may be virulent even after the onset of putrefaction, it can resist the prolonged application of a temperature of from -10° to -20° C., but, on the other hand, it is rendered inactive by one hour's exposure at 50° C. It is susceptible to the photodynamic action of methylene blue and is inactivated by ultra-violet rays. It is also inactivated by phenol, ether, chloroform, and bile.

Negri bodies. In 1903, Negri described as occurring in the nervous system in animals dying of rabies, certain bodies to which much attention has since been devoted and regarding the significance of which opinion is still divided. Negri's observations have been fully confirmed, and the occurrence of these bodies may be regarded as specific. The Negri bodies (Fig. 153¹) can be readily found in the affected nervous system by making smear preparations or sections, as described below. They vary much in size, measuring 0.25 to 25 μ , in the dog as a rule they are 4 to 10 μ . They are round, oval, or somewhat angular in outline. They are found in the protoplasm of the nerve cells and of their processes. When examined in unstained preparations, they are seen to have a sharply defined outline. Their exact staining reactions vary with the method used, but they tend to be eosinophile. They appear not to contain constituents which give the microchemical

¹ For the material from which this preparation was made we are indebted to Lt.-Col. W. F. Harvey, F.M.S.

reactions of chromatin. For the finer differentiation of the internal structure, Negri employed Giemsa's stain. With this stain and under high magnification the groundwork of the body is a pale blue; in it there appear certain round or oval formations, single or multiple, of varying size, stained pink and of homogeneous appearance (*grosse Innenformationen*). In addition, both inside these larger formations and in the general substance of the body are smaller red or violet-red granules, occurring singly or in clumps (*kleine Innenformationen*). Though the description given applies to Giemsa preparations, any eosin-methylene-blue stain is suitable for their demonstration.

Negri bodies have been found in practically 98 per cent. of cases of street rabies in dogs examined by many observers in different parts of the world. They are also found in natural rabies in other animals, and are usually present in human cases. They are likewise seen in animals inoculated experimentally with street virus. Numerous control observations on other toxic conditions of the nervous system, especially those characterised by spasms, have been made, and the consensus of opinion is that the presence of Negri bodies is a specific appearance in nerve cells and justifies a positive diagnosis of rabies.



(a)

(b)

FIG. 153. Negri bodies in hippocampus of rabid dog. Section stained by eosin and methylene blue $\times 1,000$. (a) Two bodies within a nerve cell. (b) a body showing internal structure.

fixed virus very small acidophile granules have been observed in large numbers in the nerve cells (Manouélian)

There has been great difference of opinion as to the true nature of these inclusion bodies. Negri himself regarded them as protozoa. This view was based upon their relatively constant and peculiar structure which, according to such authorities as Golgi, did not correspond to any cellular degeneration. And the fact that the nerve cells in which they are present may show no evidence of degeneration, is also noteworthy. Against their protozoal nature has been urged their absence from the brains of animals dying from fixed virus, their non-discovery in the infected *salva*, and the fact that the virus can pass through a filter. Further, in appearance they do not correspond to any known type of protozoal organism. These objections have been met with the argument that the smaller internal formations may be the infective agent in its essential form, and a modification of this view is that the Negri body is the result of a cellular change associated with invasion by these minute forms. Manouélian and Viala have regarded the Negri body as an aggregate of minute elementary corpuscles representing the causal organism, which they have named *Encephalitozoon rabiei*. By analogy with other inclusion bodies of virus diseases the view now generally adopted is that they are 'colonies' or aggregates of growth of the rabies virus in the nerve cells, but the evidence for this is not conclusive (Shortt).

The bodies occur in all parts of the nervous system, but are specially abundant in the cells of the hippocampus major and in the Purkinje cells of the cerebellum. It is in the former situation that they are generally looked for. They are apparently not so readily found, and at any rate the larger forms may be altogether absent, in animals dying from inoculation with the fixed virus (*vide infra*). Hitherto they have not been certainly found in the salivary glands or saliva of a rabid animal. In infections produced by the

Prophylaxis. Until the publication of Pasteur's researches in 1885, the only means adopted to prevent the development of rabies in a person bitten by a rabid animal had consisted in the cauterisation of the wound. A procedure was undoubtedly not without effect, and that if done within a few hours, and that if done within a few hours of the onset of the cases.

After this time, cauterisation is no longer the period of incubation, but, as will be seen, this is a most important effect. Apart from caustics, other substances have been applied to wounds with a view to inactivating the virus, e.g. 20 per cent. soap solution, tincture of iodine, etc.

The work of Pasteur, however, revolutionised the treatment of wounds inflicted by rabid animals. Pasteur started with the idea that since the period of incubation in the case of animals infected subdurally from the nervous systems of rabid dogs is constant in the dog, the virus has been from time immemorial of constant strength. Such a virus, of what might be called natural strength, is usually referred to in his works as the virus of *la rage des rues*, in the writings of German authors as the virus of *die Strasswuth*. While Pasteur's original statements regarding the constancy of virulence were probably accurate for the street dogs of Paris, it has been found that there is considerable variation in virulence under natural conditions. It is now usual to apply the term 'street-virus' to any virus derived from an animal becoming rabid by the natural mode of infection. Pasteur found that when the virus of *la rage des rues* was passed by subdural inoculation through a series of rabbits or guinea-pigs, its virulence was exalted for these animals till a constant strength (the virus *fixe*) was attained—constancy of strength being indicated by the unvarying occurrence of paresis on the sixth day. It may be noted here that the fixed virus while of high virulence on subdural injection is usually non-virulent on subcutaneous inoculation, its neurotropism is apparently intensified. Pasteur also elaborated a method by which the exalted virus contained in the spinal cords of rabbits could be attenuated. This was done by drying the cords in air over caustic potash (to absorb the moisture), the diminution of virulence being proportional to the length of time during which the cords were kept. Accordingly, by taking a series of such spinal cords kept for various periods of time, he was able to obtain vaccines of graded virulence. He found that, commencing with the subcutaneous injection of attenuated virus, and following this up with injections of more virulent material, he could immunise dogs against subdural infection with a virus which, under ordinary conditions, would certainly have caused a fatal result. Pasteur then applied himself to find whether the comparatively long period of incubation in man could be taken advantage of to immunise against the disease and thus to produce active immunity before its gravest manifestation developed.

In the earliest case treated the first injection was emulsion of rabbit's cord dried for fourteen days, and this was followed by injections of cord containing less attenuated virus. Treatment was continued over nine days, the last injection being one of emulsion of cord dried for only one day, i.e. containing approximately virus *fixe*. The patient never manifested the slightest symptom of hydrophobia. Other similarly favourable results followed, and since then immunoprophylaxis has been almost universally adopted. It is probable that apart from the attenuation produced by desiccation, the rabies virus after repeated passage in rabbits becomes less virulent to man.

A modification in the procedure was introduced later in serious cases, such as multiple bites from wolves, extensive bites about the head, and cases which came under treatment at a late period of the incubation stage. In

these the immunisation was expedited, an emulsion of cord of one day's desiccation being injected on the third day of treatment.

The method used in the Pasteur Institute of Paris is substantially the same as the original method, though certain modifications have been introduced as regards the preparation of the vaccine, its dosage and virulence (see Viala). Methods, however, vary in different institutes. The most important modifications which have been adopted are (1) the substitution by Högyes of cords subjected to decreasing periods of drying, and (2) the use of material from the brain and medulla of rabbits infected with fixed virus and the inactivation of the virus in these tissues with phenol (Semple) or ether (Alivisatos, Hempt). It is difficult from the available statistics to make any exact comparisons among the different forms of vaccine as regards their relative immunising value. Semple's method is now widely used. It is stated that in cases treated by the modified methods, certain symptoms sometimes following the original treatment, the gravest of which may be the occurrence of paralysis ('neuroparalytic accident'), are not so frequently observed. This, according to Harvey and McKendrick, may be due to the fact that a smaller amount of nerve tissue is injected in the Högyes method and also that phenol destroys the toxic property of nerve tissue. Stuart and Kinkorian, however, have recorded such neuroparalytic accidents following the administration of phenolised vaccine. They advance the theory that the nerve substance of antirabic vaccines contains a cytotoxin to which certain persons are peculiarly susceptible, but the causation of neuroparalysis is quite obscure.

virt

phenolised virus-containing material with a specific antiserum (Lemon) has also been shown that infected dog's brain can be inactivated by ultraviolet radiation, and such material under experimental conditions is a highly efficient immunising agent (Websters and Casals).

The efficacy of rabies vaccination has been generally accepted and the very low mortality in vaccinated persons (under 1 per cent.) appears to justify this method of prevention. A number of careful statistical reviews on the subject have been published by McKendrick, the data being collected from institutes in different parts of the world. In his review published in 1938 records were given of 793 663 cases treated with various forms of vaccine; among European cases the mortality was 0.83 per cent.

McKendrick was unable to establish any significant difference between the various methods of vaccination, no one being definitely superior to another. At one time the low mortality in vaccinated cases as compared with the mortality in untreated persons, 6 to 16 per cent., was cited as significant evidence of the value of immunoprophylaxis. It must of course be remembered that many persons are vaccinated who have been bitten by dogs not proved to be suffering from rabies, but records of mortality in untreated cases have likewise included persons who may have been bitten by non-infective animals. Further, the recorded figures of mortality among unvaccinated persons have been exceedingly variable, ranging from 1 or 2 per cent. to 35 per cent.; and the lack in recent years of any satisfactory statistics of mortality in untreated persons bitten by animals proved to be infected, makes it difficult, if not impossible, to assess the true prophylactic value of immunisation.

Experimental evidence has been provided by the work of Harvey and

Acton. Using monkeys, they obtained six survivals among thirty-four animals immunised before inoculation, whereas of an equal number which either were untreated or received normal brain suspension before inoculation, only one survived. It has also been found by others that vaccine given in repeated large doses may protect against subsequent subcutaneous or intramuscular injection of virulent virus (see Shortt *et al.*, Covell *et al.*, Webster). Certain workers have indicated, however, that it is difficult to protect an animal after it has been exposed to infection. Thus in spite of the universal practice of

cast on its
(see Rhodes)

required than most of those at present available (*vide supra*). They recommend the evaluation of the virus to be used in preparing vaccine by testing the lethal dose on intracerebral injection of mice. The immunising power of vaccines prepared from different strains of virus was found to vary, in this respect the Pasteur strain was superior to others tested.

Vaccination of dogs as a measure of control of the canine disease has been advocated and practised to some extent, but the immunity produced is probably of comparatively short duration, about six months, and there is insufficient evidence of the practical results of the procedure.

As has been seen above, Pasteur regarded the vaccine prophylaxis of rabies as depending on active immunisation. An 'interference' effect (p. 533) between the fixed virus and the street virus may explain the mechanism of the protection (see Rhodes).

Antirabies serum. Tizzoni and Centanni attenuated rabies virus by submitting it to peptic digestion, and immunised animals by injecting gradually increasing strengths of such virus. Both prophylactic and curative effects were obtained experimentally with the serum of the immunised animals. Marie obtained a similar serum by subcutaneous injection of sheep with virus *pre*, this prevented the occurrence of the disease when a mixture with the virus was injected into susceptible animals. This serum has been used to supplement the ordinary Pasteur treatment. Powerful virus neutralising antisera can be developed by hyperimmunisation of sheep or rabbits e.g. by inoculation with phenolised vaccines followed by the living virus. In guinea pigs and mice the therapeutic effect of such antiserum is most marked when it is injected intramuscularly at the same site and immediately after inoculation with the virus (Habel).

Laboratory Diagnosis. *Demonstration of Negri bodies.* The suspected animal is isolated and kept under observation to ascertain whether typical symptoms of rabies are present or develop. It should not be killed for further investigation until it reaches the paralytic stage, as otherwise Negri bodies may not be demonstrable in the brain. Survival for ten days of a dog suspected of rabies infection would contra-indicate rabies, as the disease is invariably fatal. When the animal has been killed or has died the brain is removed. It is placed with the vertex uppermost, and the upper parts of one hemisphere are removed in thin horizontal slices till the anterior part of the lateral ventricle is reached. The roof of the ventricle is then cut away with a probe-pointed bistoury, the hippocampus major is recognised as the laterally arched ridge which forms the floor of the ventricle. This should be transversely incised and thin slices removed for the making of smears and sections. A portion should also be taken from the cortical grey matter of the cerebrum and from the cerebellum. Smear preparations may be made as follows: a thin portion of nervous tissue is placed on a glass slide towards one end, on

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methylen-blue-eosin combination may be used, but Mann's stain or Lépine's method is recommended.

Animal inoculation. In addition to microscopic examination, a small piece of the medulla, hippocampus major, or cord of the suspected animal should be taken with aseptic precautions, emulsified in a little sterile 0.85 per cent. sodium chloride solution, and injected by means of a syringe beneath the dura mater of a rabbit. Material contaminated with bacteria should be inoculated intramuscularly. In rabies in the rabbit, symptoms of paresis usually occur in from six to twenty-three days, and death in fifteen to twenty-five days. In this way conclusive evidence is afforded of the presence of rabies infection in the suspected animal. Mice may also be used for the inoculation test (*vide supra*).

When the material for inoculation has to be sent any distance it is best to pack the head of the animal in ice. The virulence of the virus is preserved by placing tissues in glycerol.

PSEUDO-RABIES (MAD ITCH)

This is an acute infective disease occurring in various animals, e.g. cattle, in Europe and America and characterised by intense itching. Though named pseudo-rabies it bears only a superficial resemblance to true rabies. The disease has been studied by a number of workers (Ajekszky, Remlinger and Bailly; Shope, Hurst; and others). The condition is highly fatal in some animals and runs a rapid course after a short incubation period, paralysis, and death. The course of the disease, however, may be variable.

The disease is transmitted from infected animals to man, and from man to man. Histological changes are observed in the spinal cord and spinal ganglia, these consist mainly of cellular degeneration. The disease has been proved to be due to a filterable virus and can be reproduced by experimental inoculation in a variety of animals including the rabbit. The virus has been distinguished from that of rabies. It is present in the nervous system, internal organs, and in the blood. By filtration through graded collodion membranes the virus particles have been estimated to be 100 to 150 m μ in diameter. The virus has been cultivated in tissue cultures and chick-embryo medium. Immunisation of animals yields an antiserum which inactivates the virus.

SCRAPIE

This is a prevalent disease of sheep in this country and Europe. The incubation period is remarkable, varying from one to two years and the onset is gradual. Symptoms consist of nervousness, tremor, and incoordination. The disease may be general or local, and may last for a few months or years.

The disease is transmitted from infected animals to man, and from man to man. The brain and spinal cord tissue intracerebrally and by other routes, has been shown to be filterable. The experimental transmissibility of the disease and the filterability of the infective agent have also been demonstrated by workers at the Institute of the Animal Diseases Research Association of Scotland (see Greig).

EPIDEMIC POLIOMYELITIS

While the occurrence of 'infantile paralysis' (Heine-Medin disease) of sudden onset, and affecting especially one or more limbs, has been known since the earliest times, it is only coincident with the modern developments of neurology that the most prevalent type has been recognised to be associated with degenerative and inflammatory changes which are specially concentrated in the anterior cornua of the spinal cord. Though the disease chiefly attacks children, older subjects are also affected, and sometimes the disease is fatal. The disease is usually sporadic, but it is of most frequent occurrence in certain localities, and occurs in certain seasons of the year.

Sweden during the last century, but in other countries similar epidemics, limited or extensive, have come under notice. Thus in New York in the summer of 1907 an outbreak of probably over 2,000 cases occurred, 762 of which were carefully investigated by a special Commission, and it is from their work that much of our basic knowledge of the disease has been derived, and many facts regarding its infective nature have been definitely established. An even more serious epidemic took place in New York in 1916 and major outbreaks were recorded in 1931 and 1933.

Clinically, the onset of the condition is marked by more or less pronounced fever with general malaise and symptoms suggesting infection of the upper respiratory or the gastro-intestinal tract and followed after a few days by signs of muscular paresis or paralysis, as a rule, in only a relatively small proportion of cases does death result, though there is a great variation in the mortality in different outbreaks. When recovery occurs, many of the paralytic symptoms may pass off, but generally there remains evidence of definite permanent injury to the motor functions of the nervous system. The early lesions consist in a local or general leptomeningitis with pronounced emigration of polymorphonuclear leucocytes and lymphocytes into the perivascular spaces, the existence of which is reflected in the appearance of such cells in moderate numbers in the cerebro-spinal fluid. In the later stages the cells present are mainly lymphocytes. In the cord the inflammatory condition is usually marked in relation to the arterioles of the anterior commissure, especially in the cervical and lumbar regions, and thence passes along the vessels into the anterior cornua, which show intense hyperæmia with cellular infiltration of the perivascular spaces, and ultimately thrombosis or rupture of the vessels may result. The nerve cells may undergo degeneration and necrosis and become the prey of neuronophages, and secondary motor nerve degeneration may follow. Such a pathological picture, however, is not confined to the grey matter nor indeed to the cord, as similar changes have been observed in the brain. The recognition of this has widened the whole conception of the disease, and various clinical types besides the classic anterior poliomyelitis are now recognised. These depend partly on variations in the severity of the condition, partly on the disease being concentrated in a particular part of the nervous system. These less common types probably include many cases described as the acute ascending paralysis of Landry and acute bulbar paralysis, cases characterised by acute meningitis or encephalitis, cases of rapidly developing ataxia, and even cases simulating peripheral neuritis. It has been supposed that the death of nerve cells is a consequence of interference with their nutrition following the vascular lesions described above. But according to Hurst and others, if monkeys infected with a highly virulent virus are examined at a suitably early stage of the disease, marked degeneration of the nerve cells can be demonstrated without any associated vascular lesions or cellular exudate. Accordingly this is to be regarded as the initial lesion.

Experimental Transmission. The infectivity of the disease was established by the work of Landsteiner and Popper in Vienna, who in 1909 succeeded in producing the disease in monkeys by the intraperitoneal injection of an emulsion of the spinal cord of a child who had succumbed on the fourth day of illness. Similar observations were made in the same year by Flexner in New York, who found that if for intraperitoneal injection intracerebral inoculation was substituted, positive results were more uniformly produced, and the brain and cord of the infected animals were infective for other monkeys, the incubation period being from four to thirty-three days. Both anthropoid apes and lower monkeys, e.g. *Macacus rhesus*, are susceptible, and the conditions resulting from inoculation are clinically and pathologically identical with those observed in man. It has been found that *Macacus cynomolgus* is specially susceptible to direct inoculation from human cases.

The infective agent is readily filterable through the various filters which arrest bacteria, but in infecting monkeys from a human case it is advisable to commence with an unfiltered emulsion of the central nervous system, for filtered emulsions possess much less virulence, however, after passage through a few monkeys it is found that filtration has little effect in diminishing the number of successful inoculations, the virus being now so potent that

0.001 to 0.01 c.c. of an emulsion of material from the central nervous system in distilled water will produce the disease when injected into the brain. The disease can be originated by subdural and intracerebral injection, and also by introduction into the sheath of such a nerve as the sciatic. When a nerve is injected, the paralytic symptoms usually first appear in relation to that part of the cord from which the nerve emerges. Infection has also been produced by scarifying the mucous membrane of the nose and rubbing the virus into it, and even by simply injecting it into the nasal cavities. *Macacus cynomolgus* has been successfully infected by swabbing the pharynx with virus-containing material and also by feeding. The intraperitoneal, intrathecal, cutaneous and subcutaneous routes can also be employed, but to cause the disease by intravenous injection large doses must be administered. When different strains have been compared, some have proved more virulent on inoculation by the intracerebral route than by the nasal or cutaneous, whereas with others the opposite has been the case.

Monkeys are the only animals which have been regularly and successfully infected by transmission of the virus directly from the human subject. After passage in monkeys certain strains have been successfully transmitted to the cotton rat and after further adaptation in this animal have been transmitted by intracerebral inoculation to white mice (Jungeblut *et al.*). Adaptation to rats and mice is associated with loss of virulence to monkeys. Jungeblut and his co-workers have also succeeded in adapting a strain to guinea-pigs after passage in cotton rats and white mice, a paralytic disease resulting in the inoculated animals. More recently it has been reported by Durand that the Syrian hamster and certain local rodents in Tunisia are susceptible to experimental inoculation with the poliomyelitis virus.

Properties of the Virus. The discovery was made independently by Flexner and Lewis, and by Landsteiner and Levaditi, that the virus could pass through earthenware filters (e.g. Berkefeld V or N) and it was concluded that the causal agent must be very small. Observations by Elford and

embryo brain and that after serial cultivation in this way a virus from a monkey could be produced by inoculation with the culture. Sabin and Ohtsky have obtained growth in tissue cultures consisting of the nervous system of human embryos.

(By using a medium of ascitic fluid containing a piece of rabbit kidney, Flexner and Noguchi reported the cultivation from the disease of 'globoid bodies', 0.2 μ in size, and considered these to be the virus units, as shown above, the virus is of much smaller dimensions and these findings have not been substantiated.)

The virus withstands glycerolation for years, and can be kept frozen at -2° to -4° C. without being affected. It also withstands from 1 to 1½ per cent phenol for at least five days, it is, however, killed by an exposure at 50° to 55° C. for one-half to one hour.

Pathogenesis of the Disease. By means of the inoculation method the distribution of the virus in the natural and experimental disease has been determined and has been found to be similar in both cases. The virus is markedly neurotropic, and is highly concentrated in the brain and spinal cord. It also occurs in the intervertebral ganglia, the Gasserian ganglion, and in the abdominal sympathetic ganglia. It is found in the wall of the pharynx, the mucous membrane of the small intestine, mesenteric glands, and in the stools. It has also been described as occurring in the nose. While it may be present in the pharynx during the first few days of the illness it

tends to persist in the stools for long periods, even for weeks or months (Trask and Paul). It is usually absent from the blood, cerebro-spinal fluid, and urine.

Flexner's views of the pathogenesis of the disease may be summarised as follows. Infection takes place through the nasal mucous membrane, a catarrh of the buccal and nasal cavities being often the first sign of the disease. In monkeys inoculated intracerebrally the virus is eliminated into the nose, and the nasal mucus is infective in human cases. When an individual is infected by the inhalation of virus-containing secretion droplets, the virus gains access to the brain along the olfactory nerve route, thus, when monkeys are inoculated by painting the infective material on the nasal mucosa, the olfactory lobe becomes infected before other parts of the brain. This finding, as well as the size of the dose required to produce infection by intravenous injection, militates against the possibility of the virus being carried to the central nervous system by means of the blood under natural conditions. There is also evidence from experimental intravenous injections that the choroid plexus (which is the source of the cerebro-spinal fluid) so long as it is uninjured, *e.g.* by inflammation, prevents the passage of virus into the subarachnoid space. It has been found that in monkeys recovered from the disease, the nasal mucosa remains infective for many months after the virus has disappeared from the central nervous system, and it has been established that in man there are chronic carriers such as exist in other diseases. As in other conditions, the carrier may not himself suffer from the effects of the infective agent which he carries. Further, the occurrence of abortive cases may constitute a means by which infection is maintained in a community. Such abortive cases are probably fairly common during epidemics.

As has been indicated, Flexner and his associates brought forward evidence from their experimental work that the virus is present in the upper respiratory passages in cases and carriers and may be discharged from the body of infected persons in the secretions of the nose and throat and, moreover, that infection takes place through the nose. Recent studies of the disease, however, have tended to emphasise the importance of infection by the alimentary tract. Thus, the virus is readily found in the stools of cases and also contacts, and it has been recovered from sewage. There is also evidence that it may be carried by flies. The possibility has to be considered of its being transmitted by milk and other food materials. There is no definite proof so far that the infection is water-borne, though the virus may survive for long periods in water. While infection may be spread by secretion droplets from the upper respiratory passages and while the virus may enter by various avenues including the nose, tonsils, and pharynx, the evidence also points to the intestinal tract as a likely portal of entry and to the spread of the disease by the same processes as intestinal infections like enteric fever and dysentery. Pharyngeal and tonsillar infection cannot, however, be excluded and it is well recognised that tonsillectomy may precipitate an attack of the otherwise uncommon bulbar form of the disease. The question arises as to what part healthy carriers may play in spreading the infection. Their importance is emphasised by the recent finding in inter-epidemic periods of the virus in the throat or stools of children who had no known contact with cases and who did not develop the disease in the ensuing year. Observations in which a contact with known cases could be precisely traced have indicated that the infective period extends from five days before to five days after the onset of symptoms and that the incubation period is as a rule 6 to 20 days, averaging 12 days (Howe and Bodian; Kessel and Moore, Gordon *et al.*)

disease manifestations are apparently essential to the establishment of immunity, as animals which have at first yielded negative results are usually susceptible to a second inoculation. Both in man and in the monkey the serum of a recovered case contains substances capable of neutralising or inactivating the virus, for if such serum be mixed with virus and incubated for a time at 37° C. the mixture becomes inoperative on intracerebral injection into monkeys. The antibodies persist in the serum in man for many years after an acute attack; and they possess this further significance, that they may be found in the so-called abortive cases where a transient illness with little or no involvement of the nervous system occurs. The only definite evidence that such a condition is due to the virus of poliomyelitis lies in the fact that subsequently the serum has the capacity of neutralising the virus. According to Jungeblut the antiviral property is more frequent and more abundant in normal individuals of blood-group B than in those of the other groups. Moreover, doubt has been thrown on the view that the presence of those antibodies in human serum necessarily signifies either a previous infection with the virus of poliomyelitis or confers protection. Thus Burnet (1940) observed in an epidemic in Australia that some children whose serum initially had a considerable antibody content nevertheless developed paralysis, also the antibodies did not increase in the course of the illness. Again, a number of patients and contacts observed over several months showed no increase of antibodies in their serum.

Not only has an immune serum neutralising properties *in vitro*, but it has been shown experimentally to have a certain effect *in vivo* when introduced intrathecally into monkeys previous to intravenous inoculation. But Howe and Bodian failed to protect chimpanzees against inoculation *per os* by giving repeated intraperitoneal injections of hyperimmune monkey serum. Trask and his co-workers on comparing the immunity produced by a number of strains, have found differences. Strains isolated from the same epidemic appear to be more closely related immunologically than those from different epidemics. The experiments of Fairbrother and Morgan indicate that it may be possible to obtain antiviral serum from actively immunised horses.

The serum of recently recovered human cases has been injected for therapeutic purposes into patients suffering from poliomyelitis (especially during the first forty-eight hours) in amounts of 35 to 120 c.c., administered both intrathecally and intravenously. Early reports suggested that paralysis was arrested by the treatment, but extended experience in America and Europe has not confirmed its value. Flexner and Stewart also recommended a subcutaneous injection of convalescent serum as a prophylactic measure, the dose being 10 to 20 c.c. according to age, and repeated four to six weeks later if required.

It has been stated by Kraus that if virus which has been completely inactivated, e.g. by phenol, is injected into monkeys they develop resistance, but the general experience is that 'dead' virus fails to immunise. Stewart and Rhoads have found that in monkeys a considerable degree of protection against intracerebral inoculation may be developed by a series of intradermal injections of the living active virus. Accordingly the use of virus attenuated by various means, such as treatment with formalin or sodium citrate, has been advocated. These preparations are unsafe however as Leake has reported twelve cases in which an attack of poliomyelitis followed their use, paralysis occurring usually about six to fourteen days after the initial injection.

The mechanism of acquired immunity to poliomyelitis virus is still to a great extent obscure, however. Howe and Bodian pointed out that the degree

of immunity following an attack in monkeys depends greatly on the extent to which the virus was disseminated in the first instance. They have suggested also that in man immunity results from processes which prevent effective amounts of the virus from reaching the central nervous system. Experiments of Rasmussen and Clark support this view. They inoculated a series of susceptible monkeys and also animals which had recovered from an attack, by applying a concentrated suspension of virus to the proximal end of the freshly cut sciatic nerve. It was found that in four out of five control animals the virus was present in the spinal cord after three to five days, but it could not be demonstrated in the cord of any of the immunised animals.

Interference. A strain of poliomyelitis virus adapted to mice and no longer pathogenic for monkeys, is able to prevent the development of disease in the latter animals when injected intracerebrally along with a virulent strain. Further, an intravenous injection of a suitable dose of the non-virulent virus given to monkeys at an appropriate interval either before or after intracerebral inoculation with the virulent strain prevents the pathogenic action of the latter. The mouse-adapted virus loses this interfering effect after heating at 75° C. (Jungeblut and Sanders).

Laboratory Diagnosis. The fact that poliomyelitis appears under a variety of clinical types makes the diagnosis difficult in many cases, particularly of mild illness. This is specially true of the meningitic type, which may be difficult to distinguish from epidemic cerebro-spinal meningitis, especially as the characters of the spinal fluid in the two diseases may be somewhat similar, and, as is known, it may often be difficult to isolate the meningo-

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nial cord removed with

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inoculate another monkey; otherwise recovery of the first animal may occur and the virus is lost (Portions of the central nervous system may be placed in 33 per cent. glycerol for transmission to a laboratory.) Paul and Trask (1941) have described a method for demonstrating the virus in the stools by inoculation of a monkey and this procedure may be used for investigative purposes.

As evidence of previous infection the demonstration of neutralising antibodies in the serum can be carried out by using a mouse-adapted strain of virus, the protection test being made in mice (see Haas and Armstrong).

Cases have occurred where the diagnosis lay between poliomyelitis and the paralytic type of rabies, and in the present stage of knowledge the susceptibility of the rabbit to the latter disease would constitute the chief means by which the diagnosis could be arrived at.

ENCEPHALITIS

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One of the most extensive was that in which lethargica is applied. But in other epidemics differences have been observed in the susceptibility of animals to experimental inoculation, as well as in the

clinical features and the anatomical changes. We have, therefore, to deal with a group of infections. Similar diseases also occur naturally among animals. Further, certain viruses which do not ordinarily produce encephalitis in man, may do so in experimental animals under suitable conditions as to virulence and route of inoculation. It should be mentioned here that in rare cases of encephalitis in infants minute granulomata have been found in the central nervous system which contained *Toroplasma*-like bodies (*q v*). The infection is thought to be acquired *in utero*.

Encephalitis Lethargica

During the spring and summer of 1918 a number of cases of encephalitis occurred in Britain which were characterised clinically by lethargy and drowsiness, often passing into coma, with moderate or no rise of temperature. A great variety of nervous symptoms were recorded—headache, epileptic fits, spastic phenomena, ascending paralysis etc.—but the most common and

marked subdural œdema. Meningitis was not a marked feature and, when it

collections of these cells in the substance of the brain. The lesions affected especially the pons, medulla, and mid-brain, degenerative changes in the oculo-motor centres were recorded. The occurrence of ophthalmoplegia suggested at first that the condition was botulism, but the symptoms of the two diseases did not otherwise correspond and no evidence of botulism was obtained. It was also suggested that the condition might be poliomyelitis of an aberrant type, but the findings differed in certain respects from those of the cerebral cases which have been observed during epidemics of poliomyelitis, and, further, there was no evidence of a concurrent prevalence in Britain of ordinary poliomyelitis. It is noteworthy that in encephalitis the cellular infiltrations in the brain have been found to consist mainly of lymphocytes, while neutrophile polymorphs are rare, whereas the latter cells are numerous in the corresponding lesions of poliomyelitis. In support of the separate identity of poliomyelitis and encephalitis lethargica was the fact that the serum from recovered cases of the latter disease was devoid of anti-infective action on the virus of poliomyelitis. The condition was not confined to Britain, an outbreak having been recorded in Austria during 1917, and in France in 1918 and the disease became widely prevalent in various parts of the world. The bacteriological findings were as a rule negative. The disease was specially prevalent in urban districts and showed an increased incidence during the first quarter of the year, which contrasted with the seasonal prevalence of epidemic poliomyelitis.

The infective nature of the condition has been generally accepted, and attention has been called to the occurrence of mild or ambulatory cases. This has raised the question of infection being spread by 'carriers', as in the case of epidemic poliomyelitis. It has been suggested that the infection enters the upper respiratory tract. The condition of so-called 'epidemic hiccup' has also been regarded as a manifestation of epidemic encephalitis. After 1921 the incidence of the disease steadily declined.

Extensive investigations have been made into the nature of the infection, but so far without conclusive results. As a result of certain experiments in which encephalitis has been reproduced in animals by intracerebral inoculation with material from the human disease (*vide infra*), the causal agent has been regarded as a filterable virus; but most attempts to transmit the disease to animals have failed. Various workers have observed minute granular structures (Da Fano's 'minute bodies') situated intracellularly in the lesions, similar to those described in experimental herpetic encephalitis. Though it has been suggested by some that these cell inclusions represent the virus, others, e.g. Cowdry and Nicholson, have regarded them as merely cell-derivatives. In the interpretation of the results of experimental transmission of the disease to laboratory animals, a serious fallacy was brought to light, namely, the occurrence of spontaneous encephalitis in certain of these animals, e.g. rabbits and monkeys, from which they may recover, though the histological lesions, in the form of round-cell infiltrations, may persist for some time.

In 1920 Levaditi and Harvier reported that they had transmitted experimentally the virus of encephalitis lethargica and demonstrated its pathogenicity for certain laboratory animals, e.g. rabbits, the lesions being the same as in the human subject, and similar results were reported by McIntosh and others. They claimed that it was filterable, and, like certain other viruses of this nature, could be preserved in glycerol, and concluded that it was conveyed to the central nervous system by cranial and other nerve routes, but was not pathogenic if injected subcutaneously, intravenously, or intraperitoneally, or if introduced into the trachea or stomach. McIntosh and Turnbull in the same year claimed to have reproduced the disease in a *Patas* monkey by combined subdural and intraperitoneal inoculation of filtered emulsion of the cervical cord, pons, and basal nuclei from a fatal human case. The animal developed convulsive attacks and ultimately became lethargic and died on the fifty-sixth day. The microscopic lesions in the brain resembled those in the human disease. From the monkey McIntosh transmitted the infection to other monkeys and also to rabbits—an acute fatal disease, however, developing only in the latter. It may be said that the successful transmissions to animals which have been reported are very few in proportion to the failures. Levaditi and his co-workers have shown that the viruses isolated by them from cases of encephalitis produce a characteristic train of symptoms which are identical with those produced by a neurotropic strain of the herpes virus. Further facts of importance are that these viruses also produce herpetic keratitis in rabbits, and that, as shown by Levaditi in cross-immunity experiments, the virus of herpes febrilis and the supposed encephalitis virus are identical. It is worthy of note that Kleener and Amoss produced encephalitis in rabbits by intracerebral injection of the cerebro-spinal fluid from a case of neurosyphilis which showed no evidence of encephalitis. It seems likely that the herpes virus, undoubtedly widely prevalent, may sometimes become generally distributed in the tissues and fluids of the body. Thus, if all the facts are taken into consideration, the possibility must be considered that the virus recovered from cases of encephalitis lethargica was the herpes virus. The view that encephalitis and herpes in the human subject are merely manifestations of the action of the same virus can hardly be accepted on the evidence available. According to McIntosh and Turnbull, in the experimental disease which they produced, the lesions consisted of a non-purulent cellular encephalitis

in encephalitis lethargica

If we exclude as inconclusive the experiments in which the sole evidence of disease in the inoculated animals has been the finding of microscopic lesions in the brain (*vide supra*), then of the many attempts to transmit encephalitis with material from the human subject very few have been successful. Attempts to cultivate the virus by various methods have also proved unsuccessful. Thus the etiology of the condition still remains an unsolved problem, and though it may be regarded as probably due to a virus, proof is wanting.

St. Louis Encephalitis

An extensive epidemic of encephalitis occurred in St. Louis, U S A , in the autumn of 1933, the highest incidence and mortality being in middle-aged and old people. The lesions resembled those of encephalitis lethargica, but at the beginning of the epidemic especially, were more severe and more extensive in the brain and spinal cord. *Macacus rhesus* monkeys were infected by combined intracerebral and intraperitoneal inoculation with emulsions of brain from cases, but were not highly susceptible, and the infection tended to die out after several passages. The lesions in monkeys resembled those in man. Mice proved susceptible to intracerebral or intranasal inoculation, and after an incubation period developed acute encephalitis which was fatal in a few days. Passage through mice could be continued indefinitely. Rabbits proved to be insusceptible. The serum of convalescent human cases or monkeys possesses the power of neutralising the virus, as tested by inoculating mice with the mixture. By such protection tests neutralising antibodies were demonstrated in 30 per cent of sera from inhabitants of a number of American cities, on the other hand, only 9.4 per cent. of 113 normal controls without known exposure to the disease possessed antibodies (Wooley and Armstrong). Thus a wide distribution of the virus was indicated. It was shown also by protection tests that the disease is immunologically distinct from poliomyelitis, encephalitis lethargica, and forms of post-infective encephalitis. The virus is related, however, to those of Japanese B encephalitis and West Nile encephalitis (*vide infra*). It measures 20 to 30 μ (Elford and Perdrau), it can be preserved by freezing, but rapidly becomes inactive in 50 per cent glycerol. It has been cultivated in the chorio-allantoic membrane of the embryo chick and in a medium containing brain tissue from the embryo mouse.

The evidence now available points to the transmission of the virus by blood-sucking insects and other arthropods, e.g. mosquitoes, such as *Culex tarsalis* and *Culex pipiens*, and the virus has been isolated from naturally infected mosquitoes. There is also serological evidence that the infection occurs in domestic fowls and it has been shown that *Culex tarsalis* can acquire the infection from infected chicks and transfer it to other birds (Hammon *et al*). The virus has further been isolated from chicken mites and is transmitted by their eggs to nymphs of the first generation (Smith *et al*). It is thus possible that the infection is derived from animal reservoirs, both birds and mammals.

Japanese Encephalitis

Large outbreaks of encephalitis with high mortality tend to occur in Japan in the late summer ('summer encephalitis' or 'B encephalitis'). A filterable virus has been isolated from cases by Kasahara and others. It is transmissible to monkeys and mice, producing in these animals on intracerebral inoculation a meningo-encephalomyelitis. It is 20 to 30 μ in size and has been cultivated in a medium of chick embryo brain. Serum of convalescent subjects neutralises the virus, and by serum-neutralisation tests it has been differentiated from the virus of St. Louis encephalitis and that of West Nile encephalitis. The virus also differs from that of the St. Louis disease in producing a severe infection in monkeys, whereas the latter is only of low virulence for these animals, moreover, the Japanese virus is virulent to sheep, towards which the other is avirulent. The evidence indicates that the infection is mosquito-borne, culicine mosquitoes being probably responsible.

(Mitamura *et al.*). Observations by Thomas and Peck also suggest that horses may be reservoirs of the infection.

Russian Spring-Summer Encephalitis

This form of encephalitis occurs in the Far Eastern parts of Russia in the spring and summer seasons (see Silber and Soloviev). A filterable virus has been isolated from the disease, monkeys, mice, and sheep being susceptible to inoculation. The virus has been shown by complement-fixation and serum-neutralisation tests to be related to that of louping-ill (Casals and Webster). Moreover, like louping-ill, it is transmitted by ticks (*e.g. Ixodes persulcatus*) and the infection in ticks is passed to a second generation by the eggs. There is evidence that forest rodents and domestic animals are reservoirs of infection.

West Nile Encephalitis

The virus of this condition was isolated in Uganda from the blood of a person with a febrile illness (Smithburn *et al.*), and proved on intracerebral inoculation of monkeys and mice to be encephalitogenic. This virus has been compared with other encephalitis viruses in serum-neutralisation tests; it shows some relationship to the St. Louis and Japanese viruses but a serological distinction can be demonstrated. The size of the virus has been estimated to be 21 to 31 $m\mu$. Serological observations indicate the presence of infection by this virus in various parts of Africa.

A virus capable of producing encephalitis in mice by intracerebral inoculation was obtained from the blood of African natives in Uganda suffering from a non-fatal febrile disease, 'Bwamba fever' (Smithburn *et al.*, 1941). Its size has been stated as 113 to 150 $m\mu$. Smithburn and his associates (1944) also isolated an encephalitogenic virus from *Aedes* mosquitoes in the Semliki forest of Uganda, it was apparently distinguishable from other encephalitis viruses. It probably originates from an animal reservoir, *e.g.* monkeys.

Australian 'X' Encephalitis

Outbreaks of this condition have been recorded in Australia, and a filterable virus has been isolated from cases. It is transmissible to monkeys and sheep, producing in the latter animal a condition which bears a close similarity to louping-ill. The investigation of this virus and the etiology of the associated disease is still incomplete. (See Cleland and Campbell; Perdrau.)

Equine Encephalomyelitis

This disease is enzootic among horses in the United States, Canada, and in some regions of South America. It has been fully established that a filterable virus is the causal agent and two immunological types have been recognised in North America, 'Western' and 'Eastern', both of which cause disease in man. A third type has been identified in Venezuela. The virus is transmissible to a variety of animals—monkeys, mice, rabbits, guinea-pigs, birds, etc. It is estimated to be 20 to 35 $m\mu$ in size (Bauer *et al.*). Cultures can be obtained in the chorio-allantoic membrane of the embryo chick. The evidence points to the natural transmission of the disease by various species of mosquito, *e.g. Culex tarsalis*.

Hammon and his associates have found neutralising antibodies for the Western type of virus in the mammals and birds and in

This would indicate that these animals are reservoirs of infection. The disease also occurs in the human subject, probably by mosquito transmission from such animal reservoirs. Vaccination against the equine disease by means of formolised chick embryo culture has been practised with some success.

Louping-ill of Sheep

This is an encephalomyelitis of sheep, occurring in the hill grazings of England and Scotland. A prominent feature of the disease is cerebellar ataxia, and destruction of the Purkinje cells of the cerebellum is a character-

inoculation. Transmission has also resulted in mice and monkeys by intranasal inoculation. The size of the virus has been estimated as 15 to 20 $m\mu$. It has been cultivated in a medium of chick embryo tissue and in the chorio-allantoic membrane of the embryo chick. All the evidence shows that the tick, *Ixodes ricinus*, is the natural vector. Occasional cases have been recorded of transmission of the virus to the human subject with a resulting mild encephalitis, and accidental infections have occurred in laboratory workers. It has been assumed that the virus reaches the nervous system by the blood stream, but in animals infected intranasally it is suggested that the virus travels by the olfactory nerves. Apparently the infection often takes a latent or inapparent form and gives rise to immunity. Another tick-borne infection, 'tick borne fever', whose cause has not been fully defined, is also prevalent in the areas in which louping-ill occurs and there is some evidence that this disease accentuates the active manifestations of louping-ill. Considerable success has attended the prophylactic vaccination of sheep against the disease. The vaccine generally used is a formolised suspension of brain tissue from an artificially inoculated sheep. Reference has been made above to the relationship of the louping-ill virus to other neurotropic viruses. (See Gordon *et al.*, Alston and Gibson, Brownlee and Wilson.)

Borna Disease (Encephalomyelitis of Horses, Cattle, and Sheep)

This condition occurs in Europe. It is due to a filterable virus, which is transmissible by intracerebral inoculation to monkeys, mice, rabbits, guinea-pigs, and fowls. Its size has been estimated as 85 to 125 $m\mu$. The histological lesions are similar to those in other forms of encephalitis and are distributed in the brain, spinal cord, and meninges, but in addition the virus seems to spread along nerve tracts from the central nervous system, producing a neuritis. In the nuclei of nerve cells acidophile inclusion bodies ('Joest-Degen corpuscles') have been demonstrated (see Nicolau and Galloway). There is no evidence so far that this virus infects man.

Encephalitis of other Animals

Virus encephalitis occurs in certain other animals, *e.g.* foxes, mice and chickens. The mouse virus, already referred to (p. 546) is of about the same size as that of poliomyelitis and the disease produced is an encephalomyelitis with changes in the anterior cornua of the spinal cord resembling those of poliomyelitis (see Theiler, Theiler and Gard). Spontaneous encephalitis of rabbits was first noted by Bull and subsequently studied by Oliver and by Twort and Archer. The condition is often associated with nephritis. It is readily transmitted both by cage infection and by experimental inoculation. McCartney, working in the United States, found that about one-half of the stock rabbits examined by him showed encephalitic lesions. Though the lesions are similar to those of a virus encephalitis, it is of particular interest that the presence of a protozoal organism can be demonstrated (Levaditi, Nicolau and Schoen). This has been classified with the microsporidia and named *Encephalitozoon cuniculi*. It can be seen in groups of twenty to forty pear-shaped bodies apparently enclosed in a cyst.

Virus 'B'

This virus was isolated from the brain and spinal cord of a human case of acute ascending myelitis following the bite of a *Macacus rhesus* monkey. In rabbits it proved to be highly neurotropic, but produced in monkeys a condition somewhat resembling generalised vaccinia (*q v*). It would appear that in monkeys this virus is responsible for an endemic infection which seldom causes obvious signs of illness (see Sabin and Wright).

Acute Disseminated Encephalomyelitis

Of late years attention has been drawn to cases of encephalitis ('post-vaccinal encephalitis') occurring in Great Britain and other countries, about a fortnight after vaccination, a number of which have proved fatal, the majority, however, recovering completely. This condition has been met with chiefly in older children vaccinated for the first time. In view of the fact that fatal meningo-encephalitis can be produced in monkeys and rabbits by intracerebral inoculation with vaccinia virus, the question has arisen as to whether the condition is causally related to vaccinia. A characteristic histological feature in the human cases who have survived for some time is the presence in the brain and cord of perivascular areas of demyelination (Turnbull and McIntosh; Perdrau). These areas are situated most commonly in the subcortical white matter, in the thalamus, mid-brain and pons, and round the ependyma of the lateral ventricles; the cord is likewise affected. A similar condition has been long known to occur sporadically after smallpox and measles, and is also met with after other febrile illnesses and after rabies vaccination, or even spontaneously. There is little to suggest that cases which have survived the acute stage develop into disseminated sclerosis. In the post-vaccinal cases, the presence of vaccinia virus in the brain has only occasionally been demonstrated, thus attempts to transmit the condition to animals by injecting suspensions of brain from fatal cases have generally failed. While the exact nature of the causal agent is undetermined, the view has been put forward that it may be a virus or toxin acting on nervous tissues whose resistance has been depressed by various conditions, one of which is vaccination. The condition has also been regarded as an allergic manifestation, and it has been pointed out that the repeated injection of normal brain tissue from the rabbit into monkeys produces a similar encephalomyelitis (Rivers and Schwentker). In post-vaccinal cases favourable results have been reported to follow an intravenous injection of 10 c.c. of the serum of a person previously successfully vaccinated.

ACUTE ASEPTIC MENINGITIS (LYMPHOCYTIC CHORIO-MENINGITIS, ETC.)

There is a well-known group of cases of acute febrile illness with symptoms of meningo-encephalitis which usually recover spontaneously in several weeks and in which the cerebrospinal fluid is sterile as regards the ordinary bacteria and shows only slight changes, some turbidity from increase of lymphocytes being the chief, while coagulum formation is rare. Also there is no focus of infection near the meninges. A proportion of such conditions have been shown to be due to virus infections.

Lymphocytic Choriomeningitis

Armstrong and Lillie first recovered the virus of this condition by passage through monkeys, the inoculum being derived from a fatal case

during the St. Louis outbreak of encephalitis. It produced in monkeys an acute 'aseptic' meningitis, the exudate in the meninges and choroid plexus being chiefly lymphocytic. Since then it has been found repeatedly in the cerebro-spinal fluid of patients with meningitis, both in America and in Great Britain (Findlay *et al*), the symptoms usually being mild and a preceding influenza-like illness often being associated. In the cerebro-spinal fluid there is a considerable increase of lymphocytes, averaging 600 to 1500 per c.mm. The virus has also been obtained where the symptoms suggested anterior poliomyelitis or encephalomyelitis (see Farmer and Janeway) and at autopsy where there was broncho-pneumonia but no lesion of the central nervous system (Smadel *et al*). The virus has been recovered from blood, urine, naso-pharyngeal secretions, and lung. Mice, when inoculated intracerebrally or by nasal insufflation, develop tremors after five to twelve days and die in one or two days with spasms of the hind legs. Passage can be maintained indefinitely. *Post mortem*, meningitis is found and there may be encephalitis, marked lymphocytic infiltration of the brain and meninges is characteristic. The virus is present in the blood and various organs as well as the central nervous system. The spontaneous disease in mice has a low fatality often, infection may occur *in utero*. Guinea-pigs are susceptible and after intracerebral injection develop meningitis and pneumonia, subcutaneous or intraperitoneal inoculation produces a febrile illness, rats are susceptible, but usually develop only a febrile disease without nervous symptoms. Specific antibodies which neutralise the virus, appear in the serum about five or six weeks after recovery in man and the complement-fixation reaction is also given (*e.g.* with suspension of spleen of an infected guinea-pig as antigen). Such antibodies are found in the blood of a considerable number of persons who have not suffered from clinical attacks, but it is not clear whether this always represents a response to subclinical infection. The virus has been recovered both from apparently normal white mice (see Traub) and grey house mice, in such carriers a fatal attack can be induced by an intracerebral injection of sterile broth. The virus has also been found in the spleens of dogs in Great Britain. As dust in the vicinity of infected mice may harbour the virus from contamination with their urine and nasal secretion, inhalation is probably a natural mode of infection. In guinea-pigs infection can occur through the unbroken skin. Under experimental conditions various arthropods can transmit the disease (Milzer). The size of the virus has been estimated to be 37 to 60 $m\mu$. It is cultivable in the chorio-allantoic membrane of the embryo chick. It is very susceptible to heat, being inactivated at 55° C. in twenty minutes, but it survives for long periods in neutral 50 per cent glycerol at 5° C. or when dried from the frozen state. Acidophile inclusions have been described in the cytoplasm of mononuclear cells in the brains of infected animals.

For diagnosis a guinea-pig should be injected intraperitoneally with 5 to 10 c.c. of blood from a case suspected to be in the early stage of the disease or with a similar amount of cerebro-spinal fluid after meningitis has appeared. If the animal develops fever it should be killed when the rectal temperature has reached 104° F. for one to two days and the spleen and brain are placed in 50 per cent glycerol in saline and kept at about 0° C. for further investigation. Mice may be injected intracerebrally with 0.02 c.c. of cerebro-spinal fluid. The complement-fixation reaction may be used for diagnosis (*vide supra*). Serological and cross-immunity reactions distinguish the virus of lymphocytic choriomeningitis from other neurotropic viruses such as those of poliomyelitis, St. Louis encephalitis, equine encephalitis, etc.

Pseudo-Lymphocytic-Choriomeningitis

MacCallum and his associates have observed two cases resembling the above described choriomeningitis, from the cerebro-spinal fluid of which they isolated a virus with characters different from that of the typical disease. It was much larger in size, 150 to 225 $m\mu$, and serologically distinguishable.

Swineherds' Disease

In this condition, which at first presents symptoms indicating an intestinal infection,

blood, urine, and in some cases the cerebro-spinal fluid, and can be transmitted to pigs, cats, mice, guinea-pigs, and rats. Experimental transmission has also been carried out in the human subject. The relationship of this virus to others has not yet been fully investigated (see Durand *et al*)

CHAPTER XXVIII

DERMOTROPIC VIRUSES—SMALLPOX AND VACCINIA, ETC. FOOT AND MOUTH DISEASE. HERPES ZOSTER. CHICKEN- POX. HERPES SIMPLEX. MOLLUSCUM CONTAGIOSUM. WARTS

SMALLPOX (VARIOLA) AND VACCINIA

SMALLPOX is a disease to which much study has been devoted owing both to the havoc which it formerly wrought in Europe, and to the controversies which have arisen in connection with the active immunisation against it introduced by Jenner. It has been long recognised that the *contagium vivum* present in the disease is not one of the ordinary micro-organisms but is of more minute size and classifiable with the filterable viruses. In the unmodified disease there are both a severe febrile illness and also a widespread eruption on the skin and conjunctivæ the latter often leading to permanent blindness. Mixed infections, especially with streptococci, tend to occur and contribute to the serious results. The common mode of infection is probably by the respiratory tract.

Jennerian Vaccination. Until Jenner's time the only means adopted to mitigate the disease had been by inoculation of a scarified area of skin with material taken from a smallpox pustule, especially from a mild case, by this means a mild form of the disease was often originated. It had previously been known that one attack of the disease protected against future infection, and that the mild attack produced by inoculation also had this effect. This inoculation method had long been practised in various parts of the world, and had considerable popularity all over Europe during the eighteenth century. Its disadvantage was that the resulting disease, though mild, was still infectious, and thus might be the starting-point of a virulent form among unprotected persons. Jenner's discovery was published when inoculation was still considerably practised. It was founded on the popular belief that those who had contracted cowpox (vaccinia) from an affected animal were unsusceptible to subsequent infection from smallpox. In the horse also there occurs a disease similar to cowpox and known as horsepox, and Jenner believed that the matter from the ulcers following horsepox, when transferred by the hands of those who dressed the sores to the teats of cows subsequently milked by them, gave rise to cowpox in the latter. This disease was thus, in his opinion, identical with horsepox, and it had its origin in epizootics of the latter. Cowpox manifests itself as a papular eruption on the teats, the papules become pustules, their contents dry up to form scabs, or more or less deep ulcers develop at their sites. From such a lesion the hands of the milkers become infected through abrasions, and a similar local eruption occurs, with general symptoms in the form of slight fever and malaise. It was this illness which, according to Jenner, gave rise to immunity from smallpox infection. He showed experimentally that persons who had suffered from such attacks did not react to inoculation of the skin with smallpox, and further, that persons to whom he communicated cowpox artificially were similarly immune. Though from the first Jennerian vaccination had many opponents, it gradually gained the confidence of the unprejudiced, and became extensively practised all over the world, as it is at the present day.

The so-called vaccine 'lymph' which contains the immunising agent is the serous exudate of the cowpox vesicle. When such lymph is used for inoculating calf from calf by passage, a continuous supply of the product is obtained; and the lymph used for human vaccination is produced by artificial inoculation of these animals. The virus is also maintained in rabbits to serve as 'seed lymph' for inoculating calves. To maintain the potency of the virus it is advantageous to pass it alternately through the rabbit and calf; and transfer from the calf to the human subject, and then from the human to the calf is also practised. Further reference is made later to the method of production of calf lymph, its purification, and standardisation. By the use of this vaccine immunity against smallpox is conferred on the vaccinated individual for a considerable period. While some strains of the cowpox or vaccinia virus in use for the production of the vaccine have originated from natural cowpox, others probably represent the actual variola virus modified by passage in calves and rabbits; this will be discussed later. The most striking evidence in favour of vaccination was first derived from its effects among the staffs of smallpox hospitals, for here, in numerous instances, it was only the unvaccinated individuals who contracted the disease. While vaccination is undoubtedly efficacious in protecting against smallpox, Jenner over-estimated the duration of the immunity. It has been noted in smallpox epidemics that whereas young unprotected subjects readily contract the disease, those vaccinated as infants escape more or less till after the thirteenth to the fifteenth years. Revaccination is therefore necessary if immunity is to continue. In certain exceptional persons, however, the protection conferred by vaccination is short-lived (Stevenson). Vaccination just before exposure to infection yields complete protection and vaccination even in the incubation period after exposure may protect or mitigate the disease. The whole question of the efficacy of vaccination was investigated in this country in 1896 by a Royal Commission, whose general conclusions were as follows. Vaccination diminishes the liability to attack by smallpox, and when the latter does occur, the disease is milder and less fatal. Protection against attack is greatest during nine or ten years after vaccination. It is still efficacious for a further period of five years, and possibly never wholly ceases. The power of vaccination to modify an attack

restores protection,
 tive according to the thoroughness with which it is performed in
 producing the typical rock

As indicated
 of the skin, the
 and young children three scratches of $\frac{1}{4}$ inch may be made, in infants
 children and adults vaccinated for the first time it is advisable to limit the
 inoculation to one scratch as it is in such cases that post-vaccinal encephalitis
 may occur (q v). In a non-immune person a papule appears after three to
 four days and becomes vesicular in six to seven days, the lesion becomes
 pustular in ten to twelve days and is surrounded by an inflamed areola; later,
 the pustule begins to heal and a dry crust forms, which is shed about the twenty-
 first day leaving a pitted scar. The lesion is very similar to the individual

observed in those recently
 proceeding
 observed in
 to the vesicular or pustular phase. In persons who have been vaccinated some years previously and in whom
 immunity has declined. In this case the papule appears more quickly than
 in the typical reaction and, though after this the lesion goes through the usual

phases, the whole process is accelerated and final healing is complete much earlier than in a susceptible person vaccinated for the first time.

The Relationship of Smallpox (Variola) to Cowpox (Vaccinia). This is a question regarding which great controversy has taken place, a subsidiary point has been the inter-relationships within the group of animal diseases, which includes cowpox, horsepox, sheep-pox, etc. There is no doubt that cowpox can be communicated to man, in whom it produces the vesicle limited to the point of inoculation and the slight general symptoms which vaccination with calf lymph has made familiar. It does not produce in man a general eruption except in very rare cases, and the local eruption is only infectious when matter from it is introduced into an abrasion. Thus loss of infectiousness by transmission through the body of a relatively insusceptible animal is a condition which is known in other diseases. Monkeys are susceptible to both vaccinia and variola. On inoculating the shaved skin by scarification a papulo-vesicular lesion results in each case, and the lesion can be indefinitely maintained in series. Further, monkeys thus inoculated with lymph from vaccinia are rendered immune against variola, and the converse holds, though Gordon found that the degree of protection produced by vaccinia against variola was rather greater than that produced by variola against vaccinia. In the rabbit, on the other hand, similar inoculation of the skin with vaccinia gives rise to the typical lesion, whereas inoculation with variola produces only slight effects. A more delicate method than inoculation of the skin for detecting the action of variola and vaccinia in the rabbit is to inject the material into the testis, an orchitis being set up in consequence. The calf is not highly susceptible to variola, and the typical lesions are not produced at first. A slight reaction may occur at the site of inoculation, and if inoculation is made from the lesion to another calf, and then continued in series, the lesions may gradually become more marked and ultimately assume the characters of those produced by vaccinia. Thereafter, the altered virus may be indefinitely transmitted. This result has been repeatedly obtained, and the animals thus treated become immune against natural vaccinia. Not only so, but on using for human vaccination the lymph from such calves, results indistinguishable from those produced by vaccine lymph are obtained, and the transitory illness which follows, unlike that produced in man by inoculation with smallpox lymph, is no longer infectious. As mentioned above, some of the strains of vaccinia virus have originated from the variolation of calves. Thus the facts available go to show not only that vaccinia confers immunity against variola, but that variola confers immunity against vaccinia. All the evidence in regard to variola and vaccinia indicates that these diseases are due to the same virus or closely related viruses, and that the differences between them result mainly from the relative susceptibilities of the two species of animals in which they occur naturally, and the modification in virulence produced by habitat in different species. Hirst has transformed the virus of variola into one with the characters of vaccinia by inoculating the skin of a monkey with human variolous material, then passing the infection first in the monkey skin, and last through a series of rabbits by intratesticular inoculation.

While it has been customary to regard the virus of natural cowpox as the vaccinia virus propagated artificially for small-pox vaccination as the same or not dissimilar to it, Hirst has shown that they differ in certain respects. His differences in the histological structure of experimental lesions can be illustrated graphically and also a qualitative difference in the heat stability of the virus is indicated.

As has been noted above, vaccination is dated to the year 1776.

produces only a local lesion ; . . . leads to the same result. But Camus showed that injection of vaccine lymph in sufficient doses may produce generalised lesions, and this has been confirmed by others. Further, McIntosh has found that cutaneous inoculation in the rabbit with a strain of vaccinia virus rendered highly virulent by passage produced frequently a general eruption with internal focal lesions. In the human subject also, after vaccination which runs the ordinary course, the virus may be present in the blood. Therefore, in view of the fact that such dissemination of the virus may occur, the question has arisen whether the encephalitis which has been observed occasionally to follow vaccination may be caused by vaccinia ; but the evidence contra-indicates this.

Pox Diseases of Mammalian Animals. Cowpox virus produces lesions in most mammals under experimental conditions. With regard to the relation of cowpox to horsepox, it is probable that they are the same disease. Both are characterised by localised eruptions, and both appear to be ordinarily transmitted by human agency. In the horse lesions may also occur in the mouth—'contagious pustular stomatitis'. Some outbreaks of cowpox have originated from the horse, but in other cases such a source has not been traced. Sheep-pox, which occurs chiefly in south-eastern Europe, is of interest, as it presents certain analogies to human smallpox. It is a highly infectious disease, characterised by a generalised eruption. Inoculation of the sheep with lymph from a vesicle usually produces only a local lesion (though sometimes there is a slight general eruption), and thereafter the animal is immune. The virus appears to be host-specific and unrelated to those of other pox diseases. Inoculation of herds has been carried out as a preventive measure and is effective, but the animals thus treated are still infectious to fresh animals. The best results are obtained by inoculating with a mixture of virus and immune serum from a recovered animal. Goatpox presents similarities to sheep-pox, but goats do not become infected during outbreaks of sheep pox, and lymph from the latter is practically without effect on the goat. Swinepox, a disease also with a disseminated eruption, is rare, the virus is not related to those of the other pox diseases. Though at one time it was thought that the viruses of these pox diseases of different mammals might be modifications of the same virus, recent work all goes to show that with the exception of the relationship of cowpox and horsepox (and also rabbit-pox) and, of course, the relationship of variola and cowpox, the viruses of pox diseases are strictly specific.

Analogous diseases occur in birds, e.g. fowls and canaries ; these will be considered later.

Alastrim. In recent times much attention has been directed to a variola-like disease which is characterised by the relative absence of constitutional disturbances and by its non-fatal character, whilst the skin lesions may be severe, sometimes of a confluent type. It is highly infectious, and epidemics have occurred practically all over the world. Various names have been applied, such as para-variola, mild smallpox, amas, and alastrim ; the last term has come into general use. There has been much controversy with regard to its relation to variola, but we can refer only to the experimental work. The results of inoculation of animals are closely similar to those in the case of variola. In monkeys the lesions are the same with the two viruses, though Gordon found that those of the alastrim virus were rather less severe. As in the case of variola, rabbits and calves are resistant to the alastrim virus or only slight lesions occur, but Blaxter found that on passage through the calf the alastrim virus became so modified that it produced the typical lesions of vaccinia. Immunity studies have brought out no marked differences. Vaccinia protects against alastrim as it does against variola, though differences in degree may be noted, and Gordon found that the antibodies in anti-vaccinal serum react also with the alastrim virus. Cross-immunity tests, however, indicate that there is some antigenic difference between the vaccinia and alastrim viruses, thus, though vaccination protects against

alastrim, cases of the latter disease have frequently been found susceptible to vaccinia on vaccination. All the evidence at present available goes to show that the alastrim virus represents a modified form or variant of the variola virus, the modification consisting mainly in the loss of toxic and lethal properties towards the human subject, while pathogenic action in animal tests and antigenic properties have been little altered.

Nature of Variola-Vaccinia Virus. Most of our knowledge of this subject is derived from studies of the virus of vaccinia, but the evidence bearing directly on the virus present in cases of variola indicates that the two viruses are closely similar or in fact identical in many respects. It has been conclusively shown that the causal agent of variola and vaccinia is not one of the ordinary bacteria. In the exudate of skin lesions bacteria are, of course, present, but these can be removed or killed without the infective

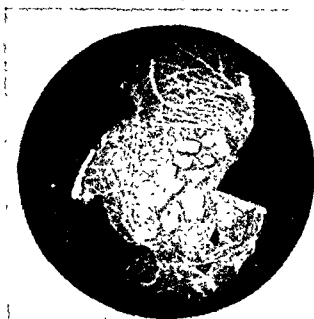


FIG 151. Plaques in the chorio-allantoic membrane five days after inoculation with vaccinia virus. (From van Rooven and Rhodes, *Virus Diseases of Man*.)

for example, obtained this. The filtration of vaccine gives divergent results. Sometimes the filtrate is infective, sometimes not. The result apparently depends upon the amount of adsorption which occurs in the filter, as it has been shown that substances like kaolin, animal charcoal, phosphate of calcium, etc., adsorb the vaccinia virus and others of similar nature. Ward has found that the virus is most readily filterable in broth with a pH of 7.6. Minute bodies were described and illustrated in the exudate of variola and vaccinia by Bust (Edinburgh) in 1886. The measurement he gave of these was 0.15μ and he demonstrated them by prolonged staining with gentian violet. He regarded these minute bodies as 'spores' of micrococci which he cultivated from the same material, but considered them to be the causal micro-organism of variola and vaccinia. There can be little doubt that the minute organisms described by Bust were the forms which were later demonstrated by Paschen

infective vaccine lymph and lesions, and designated in subsequent literature as 'elementary bodies'. It is now generally agreed that the virus particles are from 125 to 175 m μ in size by other methods generally agree with this estimate. The virus bodies have been photographed by electron microscopy and in this way have been shown to have a somewhat brick-shaped appearance. Although the virus from variola has not been cultivated *in vitro*, that from vaccinia has been found to multiply in a medium consisting of fresh minced adult hen or rabbit kidney along with fresh serum and Tyrode's solution (H. B. and M. C. Matland). Thus growth *in vitro* can be obtained in the presence of surviving tissue cells. The procedure adopted consists in mixing bacteria-free vaccinia virus with 0.3 c.c. of the minced tissue, and then adding 3 c.c. fresh rabbit serum and sufficient Tyrode's solution to make up the volume to 10 c.c. The material is distributed in 2 c.c. quantities in Carrel flasks and incubated. It may be noted that the elementary bodies appear in these cultures. Cultures can also be obtained readily in minced chick embryo and in the chorio-allantoic membrane of the developing chick. In the chorio-allantoic membrane pock-like lesions result from the growth of the virus (Fig. 15f). The variola virus can likewise be propagated in the chorio-allantoic membrane, but the pocks are smaller and slower in development.

The possibility of vaccinating against smallpox by means of a vaccinia virus cultivated *in vitro* in minced chick embryo medium has been explored by Rivers and Ward, the virus being injected intradermally. The reactions do not lead to an open sore or scarring as in Jennerian vaccination. Such inoculation is capable of preventing a later infection with the same virus from calf lymph. Stevenson and Butler have also studied the production of vaccine from cultures in the chorio-allantoic membrane of the embryo chick. The virus after repeated passage in the chorio-allantoic membrane produced skin reactions in rabbits and monkeys, which developed more slowly than those produced by calf lymph. They found also that 0.5 per cent. phenol could be added to the vaccine material without loss of antigenicity. Inoculation with the vaccine produced immunity to vaccinia virus from the calf. According to Lehmann, calf lymph cannot be replaced by culture vaccine on account of variations in potency in the latter when continuously cultivated and lack of knowledge of the duration of immunity produced by it. The method calls for much further investigation and at present no statement can be made regarding its effectiveness as a practical substitute for the older procedure.

The virus is comparatively thermolabile, its pathogenic properties being practically destroyed on exposure for an hour at 55° C. though it has still some antigenic power (Gordon). It is readily destroyed by various antiseptics, but in this respect its behaviour corresponds to other filterable viruses rather than to bacteria. For example, provided the material is kept below 0° C., it persists practically unchanged in 50 per cent. glycerol and in 10 per cent. ether, its resistance to the former being taken advantage of in the preparation of vaccine lymph for preventive inoculation. Gordon has found that the virus both in vaccine lymph and in variolous material is very susceptible to the action of potassium permanganate, being readily destroyed by a solution of 1/10,000. In fact, this substance is virucidal in higher dilutions than is carbolic acid or even corrosive sublimate. The virus when dry, as in variolous crusts, remains infective for long periods.

Inclusion and elementary bodies. Certain peculiar structures are to be found within the epithelial cells of the smallpox and vaccinia lesions both in the natural disease and in that experimentally produced, e.g. in the cornea of the rabbit, which is a suitable site for study. They have been specially investigated by Guarneri, Councilman, Ewing, Prowazek, and others. They

are usually globoid, measuring about 1 to $1\frac{1}{2}\mu$ in diameter; sickle-shaped and other forms are also met with. They are contained within the epithelial cells, often lying near the nuclei. There is no doubt that these structures, or 'Guarnieri bodies' as they are frequently called, are a feature of the lesions, but there is no evidence that they are protozoal, as supposed at one time. They appear to belong to the same class as the inclusion bodies occurring in other virus diseases. With Mann's stain they tend to be acidophile, with certain other stains basophile forms are observed. In variola intranuclear acidophile bodies have also been found, but these seem to be absent from the lesions in vaccinia.

Reference has already been made to the 'elementary bodies' which are found in the lesions and exudate of smallpox and vaccinia. These are rounded coccoid structures, 0.15 to 0.2μ in diameter and are demonstrable by Paschen's stain or by prolonged staining with Giemsa's solution which colours them red or purple (Fig. 155). It is possible that staining gives them artificially the appearance of being larger than they really are, and this is suggested



FIG. 155A. Film from culture of vaccinia virus in chorio allantoic membrane showing elementary bodies stained by Paschen's method, each division of scale = 1.14μ $\times 1,387$.

(From photographs by Prof. C. E. van Rooven.)



FIG. 155B. Film from culture of staphylococcus and *B. coli* mixed. Stained by same method as A and photographed with same magnification for comparison.

by comparisons with photographs taken by ultra-violet light (Barnard) and estimates of the size of the virus particles by filtration through collodion membranes. As mentioned above they appear to be brick-shaped when photographed by electron microscopy (Figs. 156, 157), in this respect differing from the spheroidal elementary bodies seen in certain other virus diseases, e.g. varicella. These bodies can be readily concentrated by high-speed centrifuging (e.g. 15,000 revolutions per minute), and by this means have been obtained in an almost pure state. From the centrifuged deposit a suspension of the bodies can be prepared which yields a specific agglutination reaction with an anti-vaccinal serum, behaving like a bacterial suspension when acted on by a homologous antiserum (Ledingham). According to Goodpasture and his associates the Guarnieri inclusion bodies consist of aggregates of elementary bodies. The corresponding inclusion body of fowlpox (Bollinger body) has also been shown to consist of elementary bodies similar to the minute bodies (Borrel bodies) seen as free particles in the lesions and exudates. It may be assumed, therefore, that in these pox diseases the inclusion bodies are 'colonies' of growth of the elementary virus units in the cell cytoplasm. Eisenberg-Merling has described a developmental cycle of the virus in the infected cells, but the significance to be attached to the

observation—

hydrate, with riboflavin and biotin and also mineral substances in small amount. The postulate that these bodies are organismal is a reasonable one.

Immunity and Antibodies. Various facts have been mentioned above with regard to protection afforded by cutaneous inoculation, but it has been shown that immunity can also be produced in animals by introducing the virus of vaccinia by various routes—subcutaneous, intravenous, etc. Gordon studied further the passage of the virus through intact surfaces, and found that the nasal mucosa is the most permeable, a catarrhal condition resulting which is attended by immunity to cutaneous inoculation. In animals

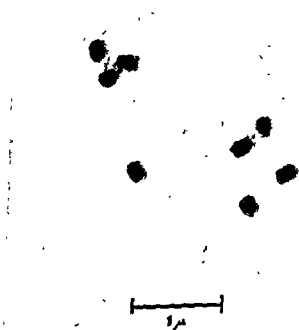


FIG. 156. Electron micrograph of Vaccinia virus $\times 20,000$
(From the National Institute for Medical Research)

immunised by the above methods, antibodies appear in the blood of a nature corresponding in some respects to those recognised in bacterial infection—inactivating or neutralising the virus ('viricidal'), and yielding precipitation (or flocculation) and complement-fixation reactions with virus-containing material. For example, when the virus is exposed for some time to the antiserum it is bereft of its infective properties, as indicated by the loss of power to cause lesions on inoculation into the skin. It has been suggested that the union of antigen and antibody is capable of readily undergoing dissociation, since neutral mixtures of vaccinia virus and immune serum which have been kept for some time may become virulent again on dilution (Andrewes). Such apparent dissociation on dilution depends on the relative quantities of antibody and virus, in the presence of a sufficient excess of antibody reactivation of the virus does not occur when the mixture is diluted (Goyal). It is manifestly difficult to determine whether any direct viricidal or even inactivating effect occurs *in vitro* or results only when the mixture of virus and antiserum is introduced into the tissues. It may be noted that

serum heated at 55° C. is as active as fresh serum, thus if *in vitro* inactivation occurs, complement does not play any part in it. Fairbrother has shown that when mixtures of vaccinia virus and antiserum are injected intradermally neutralisation can be demonstrated irrespective of the time they have been in contact *in vitro*, this is not so on intracerebral injection, four hours' contact being necessary. He suggests that the antibody sensitises the virus to the destructive action of the tissue cells. The actual mechanism of protection by an antiviral serum and its specific antibody remains obscure (see Sabin). Whether or not inactivation of the virus depends on an interaction *in vitro* between virus and antibody, such interaction is manifested by specific flocculation and complement-fixation phenomena. The antibodies in anti-vaccinia serum give flocculating and complement-fixing reactions with

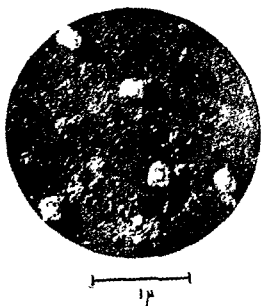


FIG. 157. Electron micrograph of Vaccinia virus shadow cast by the oblique deposition of gold (see Fig. 156) $\times 23,000$
(From the National Institute for Medical Research.)

extracts of crusts from variola and alastrim as well as with material from vaccinia. It may also be added that corresponding observations have been made on the sera of patients who have recovered from smallpox. It has been found that such sera give complement fixation not only with material from the lesions of variola but also with the virus of vaccinia. Further, Schneider showed that the serum of variola convalescents had a distinct anti-infective action toward vaccinia virus. Phenomena of supersensitiveness have been observed on reinoculating with virus and here also the action of the two types is reciprocal. All these facts support the view that the vaccinia and variola viruses are closely related, if not identical, in their antigenic characters, the former being a modified form of the latter resulting from passage in the bovine species. As mentioned earlier, an anti-vaccinia serum specifically agglutinates suspensions of the purified elementary bodies. It has been shown that the antigen of vaccinia virus contains two components, one heat-labile, the other heat-stable at 70° C., and an immune serum contains separate antibodies for these antigenic factors. It appears likely that

the virus contains still further antigens. It may be stated that the virus obtained from the lesions of varicella (chicken-pox) gives no reactions with an anti-vaccinal serum, and that no cross immunity phenomena between the two viruses have been observed. Varicella is accepted as quite a distinct disease.

There is no evidence that 'killed' vaccinia virus is capable of producing immunity. It has been suggested that immunity may depend on the persistence of the virus (Douglas, Smith and Price). Thus Olitsky and Long were able to obtain the virus from the testes of immune rabbits by a method of concentration by cataphoresis long after recovery from cutaneous inoculation. On the other hand, those animals from which the virus could not be obtained were found to be no longer resistant to reinoculation.

Agglutination of chicken erythrocytes by vaccinia virus. The phenomenon of agglutination of these cells by a virus was described by Hirst in the case of influenza virus. Nagler has shown that the same effect is produced by vaccinia virus, e.g. in the form of a chorio-allantoic culture. The effect is specifically inhibited by anti-vaccinal serum; and, as in the case of influenza virus, it is suggested that this agglutination reaction may be used for the titration of vaccinal antibodies in human and animal serum.

Laboratory Diagnosis. For the diagnosis of variola and particularly its differentiation at an early stage from varicella some value attaches to the results produced on inoculation of the rabbit's cornea with the contents of the skin lesions (Paul's test). The cornea is lightly scarified and the material (which if previously dry is suspended in a drop of saline) applied to it. After thirty-six to forty-eight hours if the material has been derived from a case of variola, small, clear, bubble-like elevations have developed on the cornea, which are best seen as opaque white spots when the eye is enucleated (the cornea being then cleansed of any adherent blood or secretions) and placed for a short time—about a minute—in a mixture of 2 parts saturated watery solution of corrosive sublimate with 1 part of absolute alcohol. Vaccinia causes an appearance which is similar, but more marked; but varicella and other infections produce nothing likely to be mistaken for it. The value of this procedure has been verified by Ungermann and Zuelzer and others.

The work also of Gordon and of Burgess, Craigie and Tulloch on the flocculating action of an anti-vaccinal serum obtained from rabbits when mixed with extracts of the skin crusts from cases of variola and alastrim, has shown that this is a specific test which possesses considerable value for distinguishing these conditions from varicella, extracts of the crusts in varicella yield a negative reaction. The crusts from the case are thoroughly dried in a desiccator and then ground to a powder in an agate mortar; the powder is weighed, triturated with normal saline, allowed to stand for an hour and then centrifugalsed. Varying dilutions of the extract so obtained are prepared, ranging from 1 in 100 to 1 in 800 (in terms of the concentration of the crust material from which the extract has been obtained). To these are added varying dilutions of anti-vaccinal serum ranging from 1 in 20 to 1 in 80 (The serum is obtained by injecting rabbits with saline extracts of vaccinia crusts from an infected animal). The mixtures are then inoculated and in a case of smallpox flocculation occurs in some of the series, i.e. in those mixtures representing optimal proportions of antigen and antibody. A control test with rabbit skin is carried out simultaneously. The flocculation reaction is carried out by Craigie and Wishart and is more sensitive than the inoculation reaction. The extract of crusts and anti-vaccinal serum are obtained as described above and

the test is carried out like other complement-fixation reactions. It should be noted that the crust extracts are sometimes markedly anti-complementary. (For details of these tests, see van Rooyen and Rhodes, Craigie and Tulloch; Craigie and Wishart.)

In early cases with a petechial prodromal eruption or with the typical

is briefly as follows: the central parts of several lesions, after cleansing with ether, are scraped with a sterile sharp-pointed scalpel, and the scrapings smeared on slides; the films are allowed to dry for ten minutes, treated with normal saline for three minutes and again allowed to dry; they are then treated with a mixture of equal parts of alcohol and ether for three minutes, when dry they can be transmitted to the laboratory for staining, which is carried out by Paschen's method. If elementary bodies cannot be detected the scrapings should be repeated every four hours while the diagnosis remains in doubt.

The application of laboratory methods for the diagnosis of smallpox may be summarised as follows: when there is a prodromal eruption or when the typical eruption is in the papular stage, the test for presence of elementary bodies and Paul's rabbit inoculation test should be carried out; when the eruption is in the vesicular stage and the vesicles are small, the microscopic test is still applicable; if the vesicles are large, the vesicle fluid may be used for Paul's test and the complement-fixation reaction; in the pustular stage, exudate or crusts may be used for the flocculation or complement-fixation test, and for Paul's test.

Cultivation in the chorio-allantoic membrane, and also testicular inoculation of the rabbit have been recommended as diagnostic tests. The former is most delicate, typical plaques appearing in three days. Either dry material or vesicle fluid in capillary tubes may be submitted for those purposes.

Preparation of Vaccine Lymph. It is not proposed to discuss fully the production of vaccine lymph, but merely to indicate broadly the processes involved, with particular reference to the purification and standardisation of the product. Calves are used and the area inoculated is the abdominal skin, after shaving and cleansing with soap and water. Vaccination is carried out by linear scratches about half an inch apart. The 'seed lymph' is rubbed into the scratches. On the fifth day after inoculation the area is cleansed with soap and water and the contents of the vesicles are collected by means of a Volkmann spoon. This material is weighed and triturated with four times its weight of 50 per cent glycerol. The 'glycerinated lymph' is then kept at -11°C . The glycerol gradually kills extraneous organisms which are invariably present in the original material. Bacteriological examinations, including bacterial counts, are made at intervals, when the number of organisms is reduced to a certain level, e.g. 5 per mgm, the lymph can be issued, provided it is free from gas-producing anaerobic bacteria and hæmolytic streptococci, and conforms to the standard of potency laid down by the Therapeutic Substances Regulations (1944, No. 370). The potency of the lymph should be such that it produces a typical vaccinal eruption, e.g. when inoculated into the skin of a rabbit, in a 1 in 1,000 dilution. The lymph must be kept at a temperature below 0°C , otherwise its potency rapidly deteriorates.

FOWLPOX

This disease exhibits various manifestations—nodular lesions in the comb and wattles, a membranous condition of the mouth or a purulent discharge from the nostrils and eyes.

(the last form being often designated 'roup'). The virus shows many similarities to that of vaccinia; and cellular inclusions ('Bollinger bodies') and elementary bodies ('Barrel bodies') have been demonstrated in fowlpox similar to those of vaccinia.

A similar disease also occurs in pigeons; but pigeons are resistant to the fowl-pox virus. Vaccination of fowls with pigeon-pox, however, produces immunity to fowl-pox and this is extensively used with success for the prevention of the latter disease (Doyle).

A pox disease occurs in canaries and the virus of this condition is also closely related antigenically to that of fowlpox.

CONTAGIOUS PUSTULAR DERMATITIS OF SHEEP

This condition is characterised by pustules on the lips and circumoral region, mucous membrane of the mouth, cornea, vulva, and other parts. The disease can be reproduced experimentally in lambs by dermal inoculation with infective exudate. Laboratory animals appear to be insusceptible. Filtrates have also been found infective, but further knowledge of the virus awaits investigation.

FOOT-AND-MOUTH DISEASE

This was the first animal disease in which a filterable infective agent was demonstrated. In 1898 Löffler and Frosch found that the fluid from the vesicular lesions of the disease, even after filtration through an earthenware filter, was still infective on experimental inoculation. The disease, which is highly infectious, attacks cattle, sheep, goats, and pigs, and exceptionally certain other animals. The characteristic manifestation of the infection is an eruption of vesicles on the feet and in the mouth. In this country the mortality is low. Cases have been recorded of its transmission to the human subject by contact with infected animals and from cow's milk.

The disease is easily reproduced experimentally in cattle, sheep, and pigs by applying infective material, e.g. vesicle fluid, to the mucous membrane on the lip or mouth. The fluid through fine filters, and the virus particles estimated diameter by filtration through gradocol membranes is about 0.1 to 12 μ . Other methods have indicated a somewhat larger size up to 25 μ or even 40 μ . The virus particles are apparently rod-shaped. The virus is

the animal develops pyrexia and a vesicular eruption on the affected foot, followed by vesicles on the other feet and on the mouth. Recovery takes place in most cases, and the animal is immune to subsequent inoculation with the same strain of virus. Rabbits, rats, and mice can also be infected experimentally, but in them the disease is less characteristic.

The virus has been cultivated in tissue cultures made from the pads, lips, tongue, and skin of embryo. It is rapidly destroyed at 53° C., but is resistant to desiccation.

defined, designated O (Oisc., ... these does not confer an effective immunity against the other ... types have also been described and there is some uncertainty whether the three recognised types are fixed or stable in their antigenic characters. In cattle immunity following infection may last for some years, and the serum of a ... type ... administered alone or along with a ... effective practical prophylaxis by immunisation has not been attained. A polyvalent antiserum produced by hyper-immunisation of cattle has been

used for prophylactic and therapeutic purposes but is of comparatively little practical value.

Reference has been made above to the extremely infectious nature of the disease, and the mode of spread has been the subject of much study. The infection is readily transmitted by direct contact, but many outbreaks occur
 survival of the virus in
 explain some outbreaks,
 transmitted by healthy
 animal carriers and even by man. It has also been suggested that wild rodents may be responsible for outbreaks in cattle, and the infection has been demonstrated in hedgehogs. It is still difficult to make any definite statement regarding all the various possible sources of infection. (See *Reports of Foot-and-Mouth Disease Committee*)

'Vesicular exanthem', a virus disease of pigs presenting a definite similarity to foot-and-mouth disease, has been studied in America. Whether it constitutes a variant type of the latter or an independent infection requires further investigation, but the virus differs in its pathogenicity from the foot-and-mouth disease virus, being non-virulent to cattle and guinea-pigs (see *5th Prog. Rep. Foot-and-Mouth Dis. Res. Committee, 1937*).

Vesicular Stomatitis of Horses. This infection bears some resemblance to foot-and-mouth disease and is naturally communicable to cattle leading also to a condition

larger than that of foot-and-mouth disease and it is also serologically different

HERPES ZOSTER

This condition is characterised by a unilateral vesicular eruption having a distribution corresponding to that of a sensory nerve, e.g. an intercostal nerve, in addition to the cutaneous lesions, inflammatory changes with lymphocytic infiltration have been described in the posterior root ganglia and the posterior cornua of the spinal cord.

It is now generally accepted that this form of herpes is infective and that it is due to a virus, but quite distinct from that of simple herpes. Using the vesicle fluid of herpes zoster, Lipschutz succeeded by corneal inoculation in producing a mild keratitis in rabbits, but passage in these animals could not be effected. He also described intranuclear inclusions in the lesions. Others have entirely failed to transmit the disease to animals, which contrasts with the easy transmission of simple herpes. In the human subject, however, inoculation of the skin with material from the vesicles in herpes zoster may reproduce the vesicular lesions and, moreover, produce immunity to re-inoculation. Such inoculation does not produce immunity to simple herpes. Elementary bodies have been found in the vesicles (Paschen, Taniguchi, Amies) and cultures have been obtained in the chorio-allantoic membrane of the embryo chick (Goodpasture and Anderson). Recent epidemiological observations and experimental work have shown that there is a relationship between herpes zoster and chicken-pox (varicella) and this is discussed under the latter disease.

CHICKEN-POX (VARICELLA)

The inoculation of animals with material from cases of varicella has, as a rule, yielded negative results, but Rivers has recorded in African vervet monkeys after intratesticular injection the local occurrence of nuclear

inclusions in endothelial leucocytes and less commonly in the tubule cells of the injected testis, which are similar to those met with in the lesions of the human disease. Elementary bodies have been demonstrated in the vesicle contents (Aragão ; Paschen ; Amies , etc.). These appear to be similar in stained preparations to the virus bodies of vaccinia, but it has been shown by electron microscopy that they are spheroidal or irregularly polygonal as compared with the brick-shaped bodies of the latter disease. The size of the virus bodies of varicella is about 145 μ (Ruska). Taniguchi and associates have obtained growths of elementary bodies in tissue cultures and in the chorio-allantoic membrane of the chick. Amies has found that

A definite epidemiological relationship has been established between varicella and herpes zoster. Thus the latter disease, occurring as it usually does in adults, may be succeeded by outbreaks of varicella in children, and many cases have now been recorded in which the two clinical conditions appear simultaneously. This relationship has been supported by the experiments of Kundratiz. He found that the inoculation of the skin of children with fluid from the vesicles of herpes zoster led in about two-thirds of the cases to the production of local vesicles ; this was followed in some by a general eruption indistinguishable from that of chicken-pox, and the development of a general reaction without a local one was also observed. Further, children who had previously had chicken-pox were insusceptible to inoculation with herpes zoster material. Also those who had reacted to the herpes zoster virus were found later to be insusceptible to inoculation with varicella material. It should be noted also that the two conditions have the same incubation period and exhibit similar histological changes. Cross agglutination and complement-fixation reactions have yielded results confirmatory of the above findings as to the close relation of the viruses of varicella and herpes zoster (Brain , Amies). However, there does not appear to be a complete antigenic identity of the viruses. It has been suggested that the viruses of herpes zoster and varicella are identical and that the clinical differences in the conditions produced depend on the individuals attacked, for example, that in the adult herpes zoster is more likely to result whereas in the child varicella occurs. Whether such identity exists or whether the viruses are distinct (though closely similar in most of their characters) must remain meantime an open question.

HERPES SIMPLEX

The etiology of simple herpes has been extensively studied, and it has been proved that the vesicular fluid of certain clinical types (e.g. herpes febrilis, cornealis, genitalis) contains an infective filterable agent or virus. Gruter first showed that the vesicular fluid of herpes febrilis, the common form of simple herpes, was infective for rabbits when inoculated on the cornea, producing a herpetic keratitis similar to herpes cornealis in the human subject, the infection being transmissible in a series of animals. The virus was rendered inactive by exposure to a temperature of 56° C. for half an hour or by being heated to the body at 37° C. for twenty-four hours. The experimental infection with vesicular fluid of a rabbit, when inoculated into the skin or cornea in man, reproduces the typical herpetic

vesicles. The herpes virus possesses practically no capacity to attack the nervous system in the human subject, though rare cases of encephalitis due to it have been recorded (see Burnet), but its neurotropism in susceptible animals may be considerable. Thus, while the experimental keratitis in rabbits may ultimately clear up without any further effects, in a certain proportion of experiments, as first shown by Doerr and his co-workers, the virus attacks the central nervous system and produces meningo-encephalitis with death of the animal. The symptoms comprise pyrexia, muscular incoordina-

condition has proved of great interest in view of its similarity to the changes in the brain in encephalitis lethargica and in animals inoculated with material from cases of this disease (*vide* p. 550). Apparently, in animals, the herpes virus may spread along motor and sensory nerves from the periphery to the central nervous system, and in the brain and cord the resulting lesions may be anatomically related to the nerves by which the virus has entered. It has been shown that the subdural inoculation in rabbits of material containing the virus either from the human subject or experimental keratitis produces a rapidly fatal encephalitis, and this condition is then transmissible in a series of animals by subdural inoculation with brain emulsion, further, corneal inoculation with brain emulsion produces a herpetic keratitis and in some cases encephalitis. A general herpetic infection in the rabbit has been produced by intravenous injection of the conjunctival secretion of an animal with experimental keratitis. Herpetic keratitis of rabbits is also transmissible to guinea-pigs (Doerr and Vochting and others), and by intracerebral injection the virus can be transmitted to mice with resulting encephalitis. The vesicle fluid inoculated by scarification on other sites in the same person may reproduce a herpetic condition. The virus has also been found in the blood and the spleen of experimentally infected animals even in the absence of generalised lesions, and in a human case of herpes simplex the cerebro-spinal fluid has been shown to be infective for rabbits (Ravaut and Rabeau).

The herpes virus may be found in the saliva of healthy persons (Levaditi, Harvier and Nicolau, Doerr and Schnabel) and inapparent infection by this virus is an exceedingly prevalent condition. Thus the occurrence of herpes may be explained by supposing that the virus is activated by intercurrent factors such as a febrile illness. It has also been shown by Dodd and others that the herpes virus causes aphthous stomatitis (acute infectious gingivostomatitis) in young children and possibly after this the infection persists in the individual for long periods, even throughout life. It has further been demonstrated that many persons have a neutralising antibody for the virus in their serum, which would indicate the persistence of the infection. It seems likely that children first become infected from adult carriers.

The virus of herpes genitalis has proved to be less virulent for the rabbit than that of herpes febrilis. Whether it is identical with that of herpes febrilis or represents a separate variety is doubtful.

A rabbit which has recovered from experimental herpetic keratitis is immune to further inoculation. At first the immunity is most marked in the cornea of the same eye but later the other cornea becomes insusceptible to inoculation, and this refractory state becomes intensified over a period of several months. After recovery from corneal infection the animal is insusceptible also to intracerebral inoculation with herpes virus. The serum of

recovered animals neutralises the virus (Zinsser and Tang; Bedson and Crawford). The serum of highly immunised animals is also capable of conferring passive immunity. Perdrau has found that cerebral immunity to the herpes virus may follow the intradermal inoculation of a neurotropic virus, though if the inoculation is deep (e.g. by deep scarification), encephalitis may result. The immunity persists for three months. According to Flexner and Amoss, strains of the virus vary considerably in virulence; those of low virulence produce encephalitis only when inoculated intracerebrally. Further, by inoculating with a mild virus, immunity to the more virulent strains may be produced.

A remarkable 'interference' phenomenon has been reported by Magrassi and more fully described by Doerr and Seidenberg.

At intervals, the length of which varies according to the site of inoculation, but which is within the incubation period, it receives an additional inoculation, this time intracerebrally with the same strain of virus. The result is that animals which are in good health and being protected between the inoculations, if the interval is too long or too short protection is not obtained and the animal dies of encephalitis. Protection is not obtained if, after correct inoculation, the interval is too long or too short. The explanation is not clear.

The virus of herpes simplex is one of the larger viruses, being 100 to 150 μ in size. Though filterable it does not readily pass even the coarsest earthenware filters and there is apparently considerable adsorption of the virus by such filters. It can be cultivated readily in the chorio-allantoic membrane of the embryo chick and in a medium of minced rabbit testis made up in rabbit plasma and Tyrode's solution. The development of the virus in the chorio-allantoic membrane is associated with visible lesions or foci, and the degree of infection of the membrane can be assessed by the number of these foci. In the epithelial cells of lesions, e.g. of the rabbit's cornea, and in nerve and glial cells of the brain in encephalitis of the rabbit acidophile intranuclear inclusions have been demonstrated, the so-called 'Lipschutz bodies' (Lipschutz, Goodpasture and Teague). They vary in size but may occupy most of the nuclear structure of the cell. The same type of inclusion can be seen in the cells of cultures.

The relationship of the herpes and encephalitis lethargica viruses has been suggested in view both of the experimental production of encephalitis by known herpes viruses and also of Levaditi and Harvier's observation that by means of brain emulsion from rabbits infected with a supposed encephalitis virus, experimental keratitis could be produced followed by typical encephalitis. In fact, Levaditi, Harvier and Nicolau concluded that the herpes and encephalitis viruses were identical, the former being only a less virulent type of the latter. This has been considered earlier in regard to the etiology of encephalitis lethargica, and it may now be concluded from all the available evidence that the herpes virus is not the causal agent of this disease.

MOLLUSCUM CONTAGIOSUM

This is an infectious disease of the skin characterised by multiple wart-like or papillomatous lesions which undergo necrosis, break down and suppurate, with later spontaneous healing. The infection is frequently spread in swim-

ming baths. Its virus etiology has been fully established. Thus, filtered material from the lesions inoculated in the skin of the human subject has been found to reproduce the disease (see Findlay). Animals are not susceptible to inoculation. Highly characteristic inclusion bodies, 'molluscum bodies', are present in the cytoplasm of the epithelial cells of the lesions. These are spheroidal or pear-shaped, measuring $20\ \mu$ to $37\ \mu$ in diameter and are acidophilic in their staining reactions. These bodies were first described by Henderson and Paterson (in Edinburgh) in 1811. Various observers have shown that the molluscum body contains large numbers of minute 'elementary' granules similar to those of certain other virus infections and this has been established by van Rooyen who has studied the inclusion bodies by micro-dissection methods. He has also pointed out that the outer covering of the body is of carbohydrate composition. The elementary bodies are about $0.25\ \mu$ in diameter (see Lipschutz); by electron microscopy they have been described as somewhat quadrangular (Fig 158). They probably represent the actual virus units and the inclusion body seems to be a developmental phase resulting from the growth of the virus in the cell.



FIG 158. Electron micrograph of elementary bodies of *Molluscum contagiosum* virus 'shadow cast' by the oblique deposition on the film of a thin layer of chromium, so that the lengths of the shadows are at least five times the heights of the particles (Williams and Wickoff) $\times 20,000$ (Prepared by Mr I. W. C. Boswell, Dept. of Physics and of Virus Infections, University of Toronto.)

For diagnostic purposes fluid from the lesion is expressed with forceps and placed on a slide, with a drop of liquor potassæ added, and examined microscopically under a cover glass. The molluscum bodies are easily seen with a dry high-power lens. If Lugol's iodine is substituted for the liquor potassæ, the bodies are stained a deep brown. A solution of 1 in 2,000 to 25,000 brilliant cresyl blue in 0.86 per cent. saline makes them even more conspicuous, staining blue inside the cell cytoplasm.

WARTS

The infectious nature of the warts which are common in childhood has been established, and the fact that the virus is filterable has been confirmed by Findlay and others, also it survives the action of 50 per cent. glycerol. A similar infectious agent has been proved to exist in specimens of *condyloma acuminatum* and probably in other papillomata, such as those of the larynx in children. In dogs and cattle infective warts are also met with. According to Findlay's observations the virus of dogs' warts is not infective for man, further, an immune serum which neutralises the human virus has no effect on that of the dog. Bodies have been described both in the cytoplasm and

which frequently become epitheliomatous (Rous and Beard)

CHAPTER XXIX

VIRUSES (*continued*)—YELLOW FEVER: RIFT VALLEY FEVER. INFECTIVE HEPATITIS AND HOMOLOGOUS SERUM JAUNDICE: DENGUE: PHLEBOTOMUS FEVER: PSITTACOSIS, ETC.: LYMPHOPATHIA VENEREUM (LYMPHOGRANULOMA INGUINALE). TRACHOMA: INCLUSION CONJUNCTIVITIS

YELLOW FEVER

YELLOW FEVER is an infective disease which is endemic in West Africa, the West Indies, Brazil, and other parts of South America. From time to time serious outbreaks take place, during which neighbouring countries also suffer, and the disease may spread to other parts of the world. In this way epidemics have arisen in the United States and elsewhere. In countries where yellow fever is endemic, though usually a few cases occur from time to time, the disease may remain in abeyance for many years and then originate apparently *de novo*. The evidence points to continuity being maintained by the persistence of a mild type of the disease ('bilious fever'). This would explain the degree of immunity which is shown during a serious epidemic by the older inhabitants. Till recently yellow fever was regarded as affecting pre-eminently urban populations, but it has recently been discovered to exist in country districts in Brazil and elsewhere. Wild monkeys and other animals are naturally infected and constitute the source from which this 'Jungle Yellow fever' is transmitted to man. The virus is transmitted by the bite of certain mosquitoes, in urban areas especially *Aedes aegypti*. This insect occurs also in regions where yellow fever is not found at present, e.g. India. Accordingly, precautions are essential to prevent the introduction by aircraft of infected insects or of patients at the infective stage.

Great variations are observed in the severity and clinical types under which the disease presents itself, and there may be confusion with leptospiral jaundice. In a typical case of the severe type after from two to six days' incubation a sudden onset in the form of a rigor occurs. The temperature rises to 104° to 105° F., the person is livid, with outstanding bloodshot eyes; there are present great prostration, pain in the back and vomiting, at first of mucus, later of bile. The urine is diminished and contains albumin. About the fifth day an apparent improvement takes place, and this may lead on to recovery. Frequently, however, the remission, which may last from a few hours to two days, is followed by an aggravation of all the symptoms. The temperature rises, jaundice is observed, haemorrhages occur from all the mucous surfaces, causing, in the case of the stomach, the 'black vomit'—one of the clinical signs of the disease in its worst form. Anuria, coma, and cardiac collapse usher in a fatal issue. The mortality varies in different epidemics from about 35 to 99 per cent of those attacked. Both white and black races are susceptible, although in the latter the disease is frequently mild. Those who have resided long in a country are less susceptible than new immigrants. An attack of the disease usually confers complete immunity against subsequent infection.

PATHOLOGICAL CHANGES *Post mortem* the stomach shows acute gastritis.

and contains much altered blood derived from hæmorrhages which have occurred in the mucous and submucous coats. The intestine may be normal, but is often congested and may be ulcerated, the mesenteric glands are enlarged. The liver is in a state of fatty degeneration of greater or less degree, but often resembling the condition found in phosphorus poisoning. The kidneys show intense glomerulo nephritis with fatty degeneration of the epithelium. There is congestion of the meninges, especially in the lumbar region, and hæmorrhages may be present. The other organs do not show much change, though small hæmorrhages under the skin and into all the tissues of the body are not infrequent. In the blood a feature is the excess of urea present, amounting, it may be, to nearly 4 per cent.

Histological changes in the liver. The changes are highly characteristic (the 'Councilman lesion'). There is a hyaline necrosis of the liver cells throughout the lobules, but most pronounced in the middle zone, along with this there is marked fatty change of the surviving parenchyma in the central and peripheral zones and in the non-necrotic cells situated among the necrotic ones. The absence of hæmorrhage, inflammatory changes or collapse of the lobular structure is also a marked feature.

An instrument of the nature of a large trocar, the 'viscerotome', has been devised by means of which specimens of liver tissue can be readily obtained *post mortem* for histological examination in order to confirm or exclude yellow fever. The method, which obviates an autopsy, is applied routinely in all cases which have died less than eleven days after onset of a febrile illness.

Transmission. Long before the actual virus of the infection came to be investigated, it was suspected that a mosquito acted as the intermediary host, and in 1881 Finlay claimed that *Stegomyia fasciata* (now designated *Aedes aegypti* or *calopus*) was the vector of the infection. In 1900 a United States Army Commission tested this hypothesis. Selecting mosquitoes which they had reared from eggs, they allowed them to bite yellow fever patients, and then to bite healthy men. Of several experiments of this nature two were successful, the first individual to be infected in this way being Dr. James Carroll, a member of the Commission, who passed through a typical severe attack of yellow fever. Experiments on a larger scale were completely confirmatory, of twelve non-immunes living under circumstances which excluded natural infection, ten contracted yellow fever after having been bitten by mosquitoes which had previously bitten yellow fever patients, all recovered. Two of the men who were thus infected had been previously exposed to contact with fomites from yellow fever patients without results (*vide infra*). The investigations of Guitéras were carried out along similar lines: of seventeen individuals bitten by infected mosquitoes, eight took yellow fever, and three of these died.

The species of mosquito used by the American Commission in Cuba was the *Aedes aegypti*. Subsequently it was shown in West Africa that several other species of *Aedes* can transmit the infection. It has been determined that a certain period must elapse after the insect has bitten a yellow fever patient before its saliva becomes infective to another subject. The length of this period depends on the temperature. It may be as short as nine days, at 22° C. it is at least three to four weeks, and at 20° C. the virus may not develop. In experiments in which the infectivity of the mosquitoes was tested by their biting monkeys, the insects when kept at 37° C. became infective in four days (Davis). The virus, however, can be shown to be continuously present in the body of the mosquito from the time it has ingested the infective blood. Infected mosquitoes retain the power of infection for a considerable

time afterwards—ninety days or longer. It has also been shown that mosquitoes may become infective after biting a patient on the first, second, or third day of the disease, but at a later period the results are usually negative, presumably because the virus is no longer present in the blood. Hereditary transmission of the virus through the mosquito's eggs apparently does not occur under natural conditions.

With regard to the possibility of communication of the disease directly from patient to patient, it was concluded after careful experiments that the disease cannot be transferred in this way, even when the contact is of a close character. In a specially constructed house seven men were exposed to the most intimate contact with the fomites of yellow fever patients for a period of twenty days each, the soiled garments worn by the patients being in some cases actually slept in by these men, not one of those thus exposed contracted the disease. These results have been subsequently confirmed.

The American Commission also transmitted yellow fever to healthy men by injecting small quantities of blood or of serum taken from a yellow fever patient at any period up till the third day of the disease. The period of incubation in this case is somewhat shorter than when the disease is conveyed by the bite of mosquitoes, the average duration in the former case being about three days, and in the latter about four days, though these times may be considerably exceeded. The blood or serum used in these experimental infections was found to be free from bacteria. That the blood is highly infective in the early stage is also shown by the fact that the disease has been contracted in the course of the manipulations incidental to making a count of a patient's blood (Low and Fairley).

JUNGLE YELLOW FEVER. Soper *et al.* have discovered that in Brazil and elsewhere in South America, yellow fever exists in sparsely populated

in the tops of trees and maintains the virus through the dry season. The infection is transmitted by the mosquitoes to monkeys and marsupials as well as to man is indicated by the presence of antibodies in their serum. The infected animals are never ill and the virus persists in their blood for a few days only. The infection can also be transmitted to monkeys by the bite of mosquitoes.

Experimental Infection in Animals. The discovery by Stokes, Bauer and Hudson, working in West Africa as members of a Commission of the Rockefeller Foundation, that certain Asiatic monkeys, especially *Macacus rhesus*, are highly susceptible to the disease led to further important advances in knowledge regarding the behaviour of the virus and immunity phenomena. Susceptible monkeys may be readily infected by subcutaneous or intraperitoneal injection of material containing the virus, e.g. the blood of a patient in the first three days of the disease, or even by smearing infected blood on the intact skin; they may be infected also by the bites of infected mosquitoes. After an incubation period, which varies somewhat according to the method of inoculation, but is usually three to four days after the bite of infected mosquitoes, marked pyrexia develops, and then albuminuria with casts and bile in the urine, and jaundice. Bleeding from the gums and black vomit occasionally occur. The infection can be maintained by passage from animal to animal by inoculation of blood or an emulsion of liver. The virulence of the virus appears to vary, but in the case of *M. rhesus* monkeys with a recently recovered virus the result was almost invariably fatal, death occurring usually six to ten days after inoculation. The pathological changes closely resemble those in the human disease. Acidophile intranuclear

inclusions have been described in the liver cells in monkeys infected with yellow fever by Torres and others (Fig. 159), although they are rare in man. The infective agent is abundant in the blood or serum of affected monkeys, since 0.0001 c.c. injected subcutaneously into a susceptible animal will usually transmit the disease. It has been proved by inoculation of monkeys that cases of mild febrile illness which could not be diagnosed clinically as yellow fever, may have the virus in their blood. As a rule the virus has not been found in the organs *post mortem* in human cases. African monkeys, guinea-pigs are susceptible, but the hedgehog

passed in series through mice by intracerebral inoculation. As a result of such repeated brain passages a 'neurotropic' strain is produced, which on inoculation into monkeys subcutaneously is found to have lost the original 'viscerotropic' characters, although a spontaneous reversion to the latter may occur (Findlay and MacCallum). The neurotropic virus causes encephalitis when injected intracerebrally into mice, monkeys (including species insusceptible to viscerotropic virus), and certain other animals. Injection of the neurotropic virus directly into the liver of monkeys rapidly restores its original viscerotropic property (Findlay and Clarke).

Properties of the Virus. Experiments with regard to the behaviour of the yellow fever virus were carried out by Reed and Carroll; they found that it was very easily killed by heat, as blood from a yellow fever patient lost its infective power on being heated to 55° C. for ten minutes (on the other hand, it is highly resistant to freezing). Blood or serum was found to be still infective after having been passed through a Berkefeld filter. This was confirmed by a French Commission, with the additional result that the virus passed through a Chamberland filter (F grade), but not through one of a finer grade (B). These facts led to the classification of the parasite among the group of ultramicroscopic or filterable viruses. By filtration the size of the virus has been estimated at 17 to 28 m μ (Findlay and Broom). Attempts to demonstrate the organism by microscopic methods have failed.

Multiplication of the virus has been obtained in a medium resembling that used for vaccinia, but containing mouse embryo instead of chick embryo, with normal monkey serum, growth is favoured by oxygenation of the culture (Findlay and MacCallum). Growth was got subsequently in minced chicken embryo tissue. The virus also multiplies in the chorio-allantoic membrane of hens' eggs.

Immunity. Recovery from yellow fever is followed in monkeys as in man by immunity. The serum of such immune individuals, even in small amounts, confers passive immunity against the virus. On the other hand, the serum of the naturally insusceptible African monkeys and of individuals who have not been exposed to infection does not possess this property, hence the presence of protective powers in the serum may be taken as evidence that

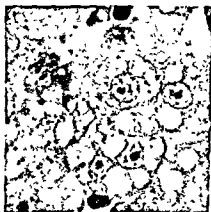


FIG. 159 Section of liver from monkey experimentally infected with yellow fever. Inclusions (Torres bodies) are seen within the nuclei of the liver cells, there is also extensive fatty degeneration with necrosis. Haematoxylin and eosin. (From a preparation by Dr G. M. Findlay) $\times 950$

Immunity remains high for at least four years afterwards. The efficacy of such vaccines has been established by extensive field trials in South America (Bugher *et al*)

RIFT VALLEY FEVER

This disease was described in 1931 by Daubney *et al* in Kenya. It affects sheep chiefly, causing a high mortality among lambs, but cattle and goats are also affected. It has been transmitted to the human subject by inoculation with a filtrate of infected tissue, a number of laboratory workers also have contracted the disease from contact with infected animal material. In man the attack resembles that of dengue. The virus is present in the blood for nine days after the onset. In sheep the characteristic lesion found *post mortem* is focal necrosis of the liver, and intranuclear acidophile inclusions have been found in the liver cells. The virus is widely distributed in the tissues, it survives *in vitro* for many months at 4° C in a mixture of oxalated blood and glycerol. Proliferation occurs in a medium of chick embryo and Tyrode solution and also in the chorio-allantoic membrane of hens' eggs. Filtration experiments indicate that the virus particles have a size of 23 to 35 m μ . Monkeys and various rodents, including rats and mice are susceptible. In mice death occurs in two to four days after inoculation by various routes. The virus can pass through the placenta to the embryo. Recovery is followed by the appearance of protective properties in the serum, and cross immunity tests have shown that the virus is distinct from that of yellow fever and dengue (Findlay). The natural vectors are probably mosquitoes. Mice can be successfully immunised with virus which has been inactivated by methylene blue and exposure to light, or by formol. It is essential that a concentrated dose of this vaccine should be injected (Mackenzie).

INFECTIVE HEPATITIS AND HOMOLOGOUS SERUM JAUNDICE

The term infective hepatitis is now applied to an illness characterised by fever and icterus which usually runs a benign course and which occurs in Great Britain and widely throughout the world. Cases may be sporadic or in epidemics. From the frequency of this disease among troops on active service it was sometimes called 'jaundice of campaigns'. Originally the infective nature of the condition was postulated on clinical and epidemiological grounds, it was classed as 'simple catarrhal jaundice' and it was recognised as not being due to a leptospira. Disease of a similar nature was then traced to the injection of products containing human serum, such as a vaccine for the prophylaxis of yellow fever, convalescent measles serum (or normal adults' serum) administered to susceptible children for the purpose of modifying or preventing an attack, and to this form the name of 'homologous serum jaundice' was given. It was also noted that jaundice which is prone to develop in patients receiving courses of treatment with organic arsenical anti-syphilitic drugs, occurred especially where there was failure to sterilise all the apparatus between successive treatments, so that there was the opportunity for one patient to receive blood from another. 'post arsenical jaundice'. The underlying lesion in those forms of jaundice has now been shown to be an acute hepatitis, which in a small proportion develops into acute yellow atrophy (see Dible *et al*, Lucké).

Since the investigation of homologous serum jaundice has afforded a clue to the causation of such icteric diseases an account of it will now be given (see MacCallum, 1946). Findlay and MacCallum and others noted the

occurrence of jaundice in persons who had received injections of yellow fever vaccine, the interval between the administration and the onset of illness varying from five weeks to seven and a half months. The virus of yellow fever could not be demonstrated in these cases; also jaundice followed vaccination of persons already immune to yellow fever as the result of a previous natural attack. The vaccine was sterile as regards ordinary bacteria and no organisms could be detected microscopically. By a process of exclusion the apparently normal human serum present in the vaccine was considered to be the vehicle of the infection. Confirmatory evidence that the jaundice in such cases occurred altogether independently of the yellow fever virus was afforded by the observation that among a group of children who had received human serum from convalescent measles patients with a view to prophylaxis cases of jaundice developed about eleven weeks later; thus out of about one hundred who received one batch of pooled serum, over one-third were affected and seven of them died (Probert; MacNalty). Other similar outbreaks have been recorded in other

regions. The presence of a virus has been demonstrated in the blood in the pre-icteric stage and early in the disease by parenteral inoculation of volunteers. Positive results have been obtained also by application of naso-pharyngeal washings intranasally, but not by ingestion of faeces or serum. The icterogenic factor passes through filter paper also it resists 0.25 per cent. phenol, exposure for an hour at 56° C. in a fluid medium, or freezing or drying for many months. In 1942 the U.S. Army suffered a heavy outbreak of 'serum jaundice' among those who had received prophylactic injections of a yellow fever vaccine containing human serum (Sawyer *et al.*). It has been shown also that in at least a proportion of cases of jaundice following administration of organic arsenical drugs an icterogenic virus is present in the blood serum.

Naturally occurring infective hepatitis was prevalent in the war 1939-45 in the Mediterranean area and the Middle East (see van Rooyen and Kirk). The incidence of this disease was much more frequent among British and American personnel than in the native inhabitants. This fact together with other epidemiological evidence, pointed to the condition being a mild endemic one which affected most severely newcomers to the area, the infection being spread from a faecal source, and flies being the vectors. The presence of a virus was proved by the production of the disease, after a incubation period of three to five weeks, in human volunteers who received *per os* faecal material from cases. Filtered serum heated at 56° C. for half an hour, when administered either by mouth or parenterally, also produced the disease. In its biological properties the virus is similar to those responsible for the other forms of infective jaundice, but it remains to be decided whether there are several such viruses affecting man or only one (see Findlay, Martin and Mitchell). The production of the disease by parenteral injection in volunteers who had recovered from serum jaundice six months earlier has been taken as evidence of a difference between the viruses (Paul *et al.*); the much longer incubation period of serum jaundice as compared with the natural form has also been regarded as indicating a difference in the respective viruses.

DENGUE

Dengue is a febrile condition presenting a fairly definite clinical picture characterised by pains in the joints and muscles, and has been long known to

have an extensive geographical distribution. The fever lasts about six days, but the period varies, there is a terminal skin eruption, which is characteristic and which does not occur in phlebotomus fever or the other febrile illnesses met with in tropical and subtropical countries. Ashburn and Craig, working in the Philippines, found the blood in this disease to be infective after filtration through a Berkefeld filter. The virus disappears from the blood after the first few days. Infectivity is retained for some days when blood containing the virus is kept at room temperature, but is destroyed in half an hour at 50° C. Epidemiological evidence points to the infection being transmitted naturally by the bites of *Aedes aegypti*, the mosquito which also conveys yellow fever, and this has been verified experimentally (see Cleland *et al*). The infectivity of the insect develops after an incubation period which is over a week, provided that the temperature is sufficiently high (20° C), and then it persists throughout life. Apparently, however, the insect may be infective at an earlier date, and this has raised the question as to whether there may not be several modes of transmission. The virus is not hereditarily transmitted in the infected mosquito's eggs. *Aedes albopictus* also acts as a transmitter, but attempts to convey the infection by *Culex fatigans* have, as a rule, failed. Recovery leads to immunity which may, however, be of short duration, as a second attack has developed less than two months after the first. Attempts to demonstrate anti-infective properties in the serum of convalescents have failed. Also an antiserum which neutralises the virus of yellow fever is without action on that of dengue. It would appear that, as in the case of yellow fever, there is liability to confusion clinically between certain types of Weil's disease and dengue and that the same applies to phlebotomus fever.

Blanc *et al.* showed that the blood or serum of guinea pigs five days after an injection of infective blood may still harbour active virus as tested by inoculation of the human subject. The virus survived similarly in various other animals. Sabin *et al.* succeeded in raising the virulence for mice by intracerebral passages through very young animals (ten to twelve days old). In early passages virulence for the human subject was preserved, but later it became reduced and the attenuated virus could be used for prophylactic vaccination. There appear to be several immunologically distinct strains. The causal agent has not been identified.

PHLEBOTOMUS (SANDFLY) FEVER

In the Adriatic littoral there occurs a disease known as "pappata" characterized by fever and pains in the muscles lasting for about three days followed by a period of protracted prostration, but very rarely having a fatal issue. Apparently the disease is liable to be introduced locally with high specific infection (see Kildes and Anders). A similar disease is found widely throughout Italy, also in other countries. It was after failure to demonstrate any connection in the blood found that the observation was made of the fact that the temperature rose during the last day of the illness, a healthy individual was killed and also 4 or 5 days later by an attack of the disease. An embryo was produced with the same result after it had been passed through a *Babesia* filter. All these facts suggest, certainly, the possibility that the disease is caused by a filterable agent. The virus has a low infectivity, at least 10 times. The virus may therefore be removed from the culture fluid by means of the capacity of the virus to pass through a virus filter, or even of water filter, although it appears that the virus may have the ability to penetrate the filter.

summer season Doerr considered there was justification for the popular view that it was associated with the bite of the dipterous fly, *Phlebotomus pappatasi*. This was borne out by the fact that on feeding such flies on a sick person, transporting them to a locality free from the disease and allowing them to bite healthy individuals, the affection was reproduced. An apparently identical disease occurs in Malta, and was first investigated by Birt under the name of 'Phlebotomus Fever'. This observer fully confirmed Doerr's results, the condition again being reproduced by infected flies, which, however, were found not to manifest infectivity earlier than seven days after biting. This last fact suggested that the causal organism may have a developmental cycle in the fly. Other phlebotomus flies are also able to transmit the disease. Infection of the insect is not hereditary, but the experiments indicated that the infection can be transmitted to insects in their breeding-grounds by the larvæ ingesting the dejecta or dead remains of the adult flies (Royal Air Force Sandfly Fever Commission, see Whittingham). Atmospheric temperature influenced the infectivity of the flies, thus insects, proved to be infective when the temperature was above 65° F., became non-infective after a fall below 60° F. The virus survives freezing for six months.

According to Shortt *et al.*, rhesus monkeys are susceptible to the virus and develop fever after inoculation with the blood of patients, also cultures can be obtained in developing hens' eggs; but these results have not been confirmed (Sabin *et al.*). Therefore the demonstration of the virus depends so far on production of the disease in the human subject; this seems to be effected most certainly by intracutaneous or intravenous inoculation. Recovery from an attack of the disease is followed by immunity which persists for some months. Immunity can be produced by injecting virus which has been inactivated by ultraviolet radiation. The serum of recovered cases when mixed with the virus may have an anti-infective action.

PSITTACOSIS AND RELATED VIRUS INFECTIONS

Psittacosis in man was so called because the sequence of disease from association with sick parrots had long been recognised clinically. Further progress was made when it was found that the infection could be transmitted experimentally from infected parrots to budgerigars and mice by filtrates free from ordinary bacteria, and that passage could be effected. Finally, by staining, characteristic masses of minute particles were demonstrated, particularly within macrophages in serous exudates and in the organs of acute cases of the infection. The individual particles vary greatly in size from coccal bodies over 1 μ in diameter, resembling gonococci, to minute bodies measuring about 0.2 μ , it is apparently due to the existence of these smaller forms that the virus can pass the coarser filters. The size and appearance of the bodies vary in the course of the infection and this has been interpreted as evidence of a cycle of development. In parrots the disease is a gastro enteritis, while in man broncho-pneumonic lesions are produced by the infection. In the case of pneumonia in which the usual causal bacteria cause pneumonia (resembling that of psittacosis) to the discovery of psittacosis. In some there is a connection with infected birds other than parrots, e.g. pigeons, chickens, fulmar petrels, hence the designation ornithosis has been suggested for the disease. In some an association has been noted with the so-called distemper of cats (Baker), where a similar virus is present, while others again are not obviously related to infections in animals. A pneumotropic virus of this nature has been recovered from mice (Nigg *et al.*;

Karr) Also, apparently healthy carriers of such viruses are common, e.g. in English pigeons (Andrewes and Mills). It has been recognised that the same strain of virus may show marked variations in virulence for a particular animal species on experimental inoculation. But different viruses have been distinguished on the basis of their varying pathogenicity by one route of inoculation as compared with another, e.g. the susceptibility of mice to intracerebral inoculation with psittacosis and their resistance to mouse pneumonia virus, whereas both viruses cause pneumonia on intranasal inoculation, while on intraperitoneal inoculation psittacosis virus tends to establish the carrier state in animals which do not die acutely, but mouse pneumonia virus fails to infect at all. Cross immunity tests have also brought out differences between members of this group of viruses, in some instances a virus produces solid immunity toward itself but not toward allied viruses, whereas others protect against both. The results, however, seem to depend to a considerable extent on the route of inoculation. Complement-fixation reactions have also been used in studying the relations of these viruses. By a combination of these various methods Beck *et al.* have distinguished three groups of such viruses responsible for pneumonia in man. It should be noted here that viruses with other characters may also be a cause of pneumonia (p. 394).

A further group of diseases clinically not resembling psittacosis are characterised by similar cell inclusions, e.g. lymphopathia venereum, trachoma, and inclusion conjunctivitis. A number of the above-mentioned viruses have been cultivated in tissue cultures and either in the chorio-allantoic membrane or in the yolk sac of developing hens' eggs. It may be said that in this group we have to deal with viruses which are closely similar in morphology, and while they may produce a variety of lesions and immunological distinctions can be established between them, nevertheless they are antigenically related (Rake *et al.*)

Psittacosis. In 1929 numerous cases appeared in the Argentine, the United States, Great Britain, and various parts of the Continent. The great majority of these were obviously related to diseased parrots recently imported, and when importation was prohibited the epidemic soon ceased. The infection occurs in wild parrots in Australia as well as in S. American birds. The characteristic lesion in human psittacosis is a widespread pneumonic consolidation, which to the naked eye may resemble that found in influenzal pneumonia, but the microscopic appearances are different. In psittacosis pneumonia there are found in the alveoli marked fibrinous exudate and some hemorrhage, with mononuclear cells, but relatively few polymorphs, in the most advanced parts of the lesion there is also extensive capillary thrombosis. Influenza bacilli and the other organisms commonly found in pneumonia are not present.

Bedson, Western and Levy-Simpson demonstrated that the disease could be transmitted to budgerigars (*Melopsittacus undulatus*) by intraperitoneal injections of sterile filtrates of the tissues of infected animals. Repeated passage has been effected but the virulence of the infection tends soon to become extinguished in these birds. Fowls are susceptible, but passage cannot be effected. Parrots can be infected by the mouth or nose as well as parenterally. Mice are also susceptible to inoculation with infective material by either the intracerebral, intraperitoneal, or intranasal route. In the last case pneumonic lesions result, and if a sufficiently dilute suspension of virus is used discrete foci of consolidation form. The age of the infection succeeds in mice, and finally a constantly fatal pneumonia. In budgerigars intraperitoneal inoculation with liver and spleen emulsion

Coles, Levinthal, and Lillie independently demonstrated the presence in the disease of minute cocci or cocco-bacillary bodies about $0.2\ \mu$ in size, stained purple to blue by prolonged treatment with Giemsa's stain; but there is great pleomorphism, the size varying from $1\ \mu$ down to minute particles. These bodies, which are Gram-negative, occur especially in the spleen and liver in affected birds, but are scanty in the blood. They are situated within macrophages, but occur also in clumps lying free; they have been seen in similar cells from the pneumonic areas in man. In infected animals a sequence of changes may be made out in the appearance of the virus particles, which Bedson and Bland have interpreted as indicating a developmental cycle. They have not been grown on ordinary media, but they multiply in tissue cultures and in the chorio-allantoic membrane etc. of hens' eggs (see Lazarus *et al.*). Their number in a tissue appears to correspond with the virulence for susceptible animals.

The virus is present in as well as in the blood and that the disease may be cor- being traceable. The occurrence of pneumonia as the chief lesion in man points to the disease being acquired by inhalation; this is supported by the production of similar lesion intratracheal inoculation tion in these animals lea-

not develop. Parrots, however, by whatever route they are infected, do not show lesions of the lungs, the organs affected being chiefly the duodenum and the liver. The virus has been found in human sputum, which suggests that the disease may be directly contagious; clinical observations are in agreement with this, but such spread appears to occur only rarely. Infective material shows considerable resistance both to freezing and drying, but is partially inactivated by exposure at 55°C . for thirty minutes, while 80°C . destroys it. Both in birds and in man the severity of the disease may vary greatly, and the evidence points to the existence among parrots of apparently healthy carriers of the virus. In rabbits intracutaneous inoculation leads to a local papular eruption resembling that which may be produced by the virus of herpes, while intracerebral inoculation causes meningo-encephalitis (Gordon). Recovery is accompanied by active immunity. Mice can be actively immunised with formalised virus (Bedson). The psittacosis virus is not influenced by an antiserum to fowl-plague virus; this observation, as well as the fact that the former is infective for mice but not the latter, indicates the separate identity of the two viruses.

Laboratory Diagnosis. In birds suggestive lesions are pericardial effusion, greatly enlarged spleen, pale liver with necrotic areas sometimes with hæmorrhagic periphery. Smears from the pericardial and peritoneal exudates, blood, lungs, air sacs, and serous and cut surfaces of liver and spleen (particularly from necrotic areas) are stained for virus bodies. Several mice are inoculated intraperitoneally with 0.5 to 1.0 c.c. of a mixed emulsion of the various tissues and also with the same material after clarification and filtration through a coarse type of bacterial filter, e.g. Berkefeld V—the latter filtration is necessary if there is a mixed infection with pathogenic four-ry shams with rification and Post-mortem material

glass beads, and is inoculated unfiltered and filtering, both peritoneal and nasal routes being used.

obtained at a late stage of the illness is usually negative, but scanty virus may be detected by giving a mouse three injections on successive days. Since viruses belonging to this group may not withstand glycerol, material for examination should be preserved below 4° C. without any addition.

Infected mice die usually in five to thirty days. If death has occurred in the first half of this period virus particles are readily found in the endothelial cells of the peritoneal and pericardial effusions; later they are scanty. Passages through further mice raise the virulence till death occurs in two or three days, virus bodies being abundant in the spleen phagocytes. If all the mice originally inoculated survive for ten days, some should be killed at this time and their spleens used to inoculate further animals. If the rest of the original mice survive for a month they should be treated similarly.

Eggs which have been incubated for ten to twelve days should also be inoculated in the chorio-allantois.

Great care should be taken with all animals and materials to avoid dissemination of infective dust and it is advisable to use the draught apparatus employed in inoculating animals with *Rickettsia* (*g.v.*). Carcases before examination should be soaked in a solution of lysol so as to wet thoroughly hair or feathers.

Staining of the virus particles is effected by Castaneda's or Giemsa's method.

LYMPHOPATHIA VENEREUM (LYMPHOGRANULOMA INGUINALE)

This disease, known also as paradenitis, climatic bubo, or Nicolas-Favre disease, which is caused by sexual contact, occurs in the male chiefly as a

there is a small lesion at the site of inoculation on the penis, which soon heals. In the female the inguinal glands may be similarly affected, but if the primary lesion is situated posteriorly on the genitalia, the rectum and anus are affected, stricture resulting, and this together with chronic swelling and ulceration of the vulva constitutes 'esthiomène', a condition which leads to persistent infectivity. Hellerstrom and Wassén transmitted the infection to monkeys by inoculation with material from affected glands. After intracerebral injection there was an incubation period of six to twelve days and then meningo-encephalitis developed which often proved fatal. Passages were successful and on transference to the human subject again the disease was reproduced (Levaditi *et al.*). Mice are also susceptible to intracerebral inoculation, and by the intranasal route pneumonic lesions result. The virus is filterable through the coarser filters. It has little resistance to glycerol. Elementary bodies have been demonstrated by Miyagawa within histiocytes, polymorphs, and glial cells. The size of the virus bodies is 125 to 175 μ . The virus grows in the yolk sac of the developing egg and such cultures are toxic for mice in virtue of an endotoxin, which is thermolabile, however (Rake *et al.*). Growth in tissue cultures has also been obtained, especially at 18° to 28° C (Sanders).

Frei showed that in cases of this disease the skin reacted specifically to the intradermal injection of pus from a bubo diluted with saline and sterilised by heating at 60° C. An area of inflammation develops, which reaches its height in forty-eight hours, this reaction is of value in diagnosis. The Frei reaction can also be obtained by injecting an emulsion of the brain of experimentally infected monkeys or mice (D'Aunoy *et al.*) or a suspension of elementary bodies prepared from infected yolk sacs, 'lygranum' (Shaffer *et al.*).

Similar antigens have been used for the complement-fixation reaction with patients' sera (see Landau). Positive results may be obtained with latent as well as active infections. Owing to the occurrence of group reactions however, a positive result does not necessarily prove infection with any particular member of the psittacosis-lymphopathia-venereum group of viruses.

Granuloma venereum (inguinale), which is to be distinguished from the above condition is a chronic disease of the genitalia, leading to permanent disability. It is widely distributed in tropical and subtropical regions. Unless treated with arsenic, it may last for years. The disease is characterized by small granulomatous papule, usually on the genitalia, ulcerates, becomes secondarily infected and spreads locally both extensively and deeply, disseminated foci may develop. In the lesions there are large mononuclear cells in the protoplasm of which are found masses of Gram-negative pleomorphic bacillary forms which are capsulate and resemble Friedländer's bacillus (Donovan bodies), but extracellular and non-capsulate forms are seen also. Before ulceration no other organisms are present in the tissues and cultures on all ordinary media are negative. Proliferation occurs in the yolk of fertile eggs, and the growth-promoting property resists heating at 60° C for half an hour (Anderson, De Monbreun and Goodpasture). Animals are not susceptible to inoculation, but the condition has been reproduced experimentally in man by injecting infected tissues or pure cultures in yolk (Greenblatt *et al*). The organism does not pass even the coarsest bacterial filters, it has considerable viability at 5° to 37° C. A sterilised culture causes an inflammatory reaction on injection into the skin of infected persons and an extract gives precipitation and complement-fixation reactions with their serum. The exact nature of this organism, which appears to be the causal agent of the disease, is uncertain, it has been named *Donovania granulomatis*.

TRACHOMA

The infective character of trachoma appears to be definitely established clinically. Halberstädter and Prowazek described in trachoma cases inclusion bodies in the cytoplasm of the conjunctival epithelium, which at their earliest stage appeared as minute coccid forms stained blue with Giemsa's solution, then red granules appeared in the middle of these, and finally only small eosinophile granules and rods, about 0.25 μ in length, were seen. The bodies are most numerous at the acute stage. The appearances are similar to those in psittacosis. But cultures have not been obtained so far and there is still discussion as to the nature of these bodies (see Julianelle). Transmission of the disease to monkeys by filtrates of material from cases has been claimed, but this is not certain. However, the virus remains infective for man after repeated monkey-passages (Bland).

Noguchi isolated from the lesions of trachoma a small Gram-negative motile bacillus with a single terminal flagellum, *Bact. granulosis*, which grows well aerobically at 30° C on agar containing defibrinated or citrated horse blood or serum, and he claimed to have produced the disease in monkeys by injecting pure cultures of this organism beneath the conjunctiva. It does not appear to be invariant. Olitsky and others have shown that a similar bacillus, *Bact. sinica* which, however, grows on ordinary agar medium, there is no clear evidence that *Bact. granulosis* plays a part in the causation of trachoma.

Diagnosis. The eyelid is everted, surface secretion wiped off the conjunctival surface, and then as much epithelium as possible scraped off with a knife, bleeding being avoided. A film of the epithelium is made on a microscope slide after the manner of a blood film. This is then stained by Giemsa's method, etc.

INCLUSION CONJUNCTIVITIS

A form of mild, persistent conjunctivitis occurring a few days after birth, but found also in adults ("swimming bath conjunctivitis"), is characterized by inclusion bodies in the epithelial cells which resemble those in trachoma, although clinically the disease is quite different. It has been found in the conjunctiva of the cervix uteri in baboons (Thygeson, Julianelle *et al*).

CHAPTER XXX

VIRUSES (*continued*)—EPIDEMIC INFLUENZA, ETC. · INFECTIOUS CORYZA · VIRUS PNEUMONIA · MEASLES · RUBELLA · DURAND'S DISEASE · INFECTIVE MONONUCLEOSIS · MUMPS · EPIDEMIC KERATO-CONJUNCTIVITIS · EPIDEMIC GASTRO-ENTERITIS · CANINE DISTEMPER · RINDERPEST · SWINE FEVER · VIRUS MYOCARDITIS · INFECTIOUS NEOPLASMS OF RABBITS · VIRUS DISEASES OF DOMESTIC BIRDS

EPIDEMIC INFLUENZA

Authentic European records over nearly two centuries show that at varying intervals there have occurred severe and widespread epidemics of a condition which, in the description of its clinical features, conforms to the syndrome now designated influenza. Such epidemics have shown a tendency to pandemic spread, and this is an outstanding epidemiological feature of the disease. Since the period of bacteriological investigation two such pandemics have occurred (1889-92 and 1918-21) and their etiology has been extensively studied. The disease exhibits an exceptionally marked degree of infectiousness and assumes a high incidence in any population in which it occurs, spreading with great rapidity. In Chapter XVIII the organism first reported by Pfeiffer as the causal agent of epidemic influenza is described and its relationship to the disease has been discussed.

The inconstancy of this organism in epidemic influenza, the fact that indistinguishable organisms may occur in various inflammatory conditions of the respiratory system, and the absence of experimental proof of its etiological relationship to the disease, led to further inquiry being made into the etiology of influenza during the pandemic of 1918 and the following years. The knowledge that had been obtained regarding the causation of certain specific infectious diseases by filter-passing viruses directed attention particularly to the question whether this epidemic disease was the result of infection by such a virus. Further, the extreme infectiousness of the condition, by analogy with other conditions known to be due to filterable viruses, seemed to favour this view of the etiology of the disease. In the early stages of the pandemic, various workers claimed to have established the existence of a filter-passer in the disease (Gibson, Bowman and Connor, Nicolle and Lebailly, Yamanouchi, Sakami and Iwashima, *et al*). Certain of these claims, however, were subject to serious criticism, though an influenza-like disease was apparently produced in susceptible persons by the injection into the nose and throat of filtrates of sputum from influenza cases.

In 1933 further light was thrown on the etiology of influenza by work at the National Institute for Medical Research, London (Smith, Andrewes and Laidlaw). During an epidemic in that year filtered throat washings obtained from patients at an early stage of the illness were found to be infective to ferrets by intranasal instillation. No other laboratory animals could be similarly infected. Illness ensued in the ferret after an incubation period of two days and showed characteristic features, namely, a diphasic temperature response, nasal catarrh, and systemic disturbances. The temperature at the onset rose abruptly, subsided on the third or fourth day and rose again on

the fourth or fifth day, later gradually returning to normal. With the rise of temperature the animals became lethargic and showed muscular weakness. The catarrh usually began on the third day with a watery discharge from the eyes and nose. The nasal discharge tended to become muco-purulent. Frequent sneezing was a prominent symptom. Signs of illness lasted as a rule for only a few days and complete recovery then ensued. Very occasionally a relapse was observed. No fatalities resulted. In animals killed during the first and second febrile periods the mucosa of the nasal passages showed acute inflammation with complete disappearance of ciliated cells and occasionally patchy necrosis of the whole thickness of epithelium. Apparently in the infected person or animal the virus propagates with great rapidity in the surface cells of the respiratory passages and also spreads rapidly from cell to cell.

It was found that the disease could be transmitted by contact or by transference of nasal washings from infected to normal ferrets. The method adopted for transmission experiments was to scrape out the turbinates and grind up the tissue with sand and then emulsify in a mixture of broth and saline. The emulsion was centrifuged and 1 c.c. of the fluid was instilled into the nose. In this way it was possible to make serial passages. No other method of inoculation proved successful and no other tissue, with the exception of the lungs, was found to be infective. Filtrates of these emulsions obtained with gradocol membranes having an average pore-size of 0.5μ invariably produced the typical disease. No bacteria were cultivated from the filtrates. Ferrets recently recovered from the illness were in all cases immune to subsequent inoculation. The serum of recovered animals also neutralised the virus when a mixture was inoculated into the nose. Normal ferret serum had no such action. Human sera, particularly those from influenza convalescents, were also found to contain neutralising antibodies for the virus. It may be noted that throat washings from healthy persons, influenza convalescents, and cases of the common cold caused no illness in ferrets.

Previous to these observations Shope in the United States had described a filterable virus in an influenzal disease of pigs, but found that it was associated with a hæmophilic organism, *B. influenza suis*. By experiment he showed that the virus *per se* produced in pigs only a mild illness, while the bacillus was inactive. On the other hand the two agents together caused the typical disease.

due to the human influenza virus. Shope later observed that influenza virus when introduced intranasally in ferrets under ether anaesthesia produced often extensive atelectasis and consolidation of the lungs.

Attempts were made by the National Institute workers to adapt the virus from ferrets to mice and this was found possible, the virus material being instilled into the nose under ether anaesthesia. After several passages in mice, the virus infected nearly all mice so inoculated and frequently led to a fatal illness characterised by pneumonia. After passage through mice the virus was still virulent to the ferret. The upper respiratory passages in the mouse did not appear to be susceptible as in the ferret. The same results were obtained with Shope's virus. It has not been possible, however, to infect mice directly from the human subject. It was estimated by filtration through Elford's gradocol membranes that the diameter of the virus bodies was from 80 to 120 m μ . Further work on the size of the virus has yielded somewhat varying results and discrepancies. According to Sharp and co-workers, who have measured the virus bodies by electron micrography, the

average size of type A is 77.6 $m\mu$ and of type B 97.3 $m\mu$. They describe type A as kidney-shaped. The virus has been shown to possess a certain degree of resistance to natural drying (Edward).

It is now recognised that strains of influenza virus differ in their antigenic characters as judged by neutralisation tests with specific antisera. Strains similar to that originally isolated in Great Britain are designated 'influenza A virus', and a B type, quite different from A, has been defined.

Both types have some variants of each have been reported.

Two factors have been identified in these viruses, one closely associated with the virus body, the other more soluble or diffusible, and it is suggested that the former is the more specific. In attempts to produce influenza in the human subject with laboratory strains, success has usually been attained with virus B, but not A, whereas the B virus is more difficult to transmit to ferrets and mice. Cases and outbreaks of otherwise typical influenza have been investigated in which it has been impossible to demonstrate transmissibility to ferrets and it has been suggested that a type of virus may exist which is non-pathogenic to animals and this form of disease has been spoken of as 'influenza Y'.

The condition of swine influenza in connection with the etiologic was apparently unrecognised in the United States—simultaneous occurrence of the human pandemic of influenza at that time, since then it has persisted. The symptoms and signs of the disease distinctly resemble human influenza. The question therefore arises whether this virus is related to strains of human origin. Disease has been produced in young pigs by intranasal inoculation with the human virus (Elkeles, Shope and Francis). The illness is of a mild type, but there may be broncho-pneumonic areas in the lungs or lobular atelectasis. According to Shope and Francis, inoculation of pigs with a mixture of this virus and *B. influenza suis* brings about a more severe illness, attended with fever and pneumonia. The National Institute workers have shown by neutralisation tests with antisera that while the antigens of the human and pig viruses have common constituents, they can be clearly differentiated. Laudlaw suggested that the virus of swine influenza is in reality the virus of the pandemic disease of 1918, adapted at that time to the pig and maintained since then in this animal. The question thus arises whether the virus of the 1918 pandemic was antigenically different from those now present in influenza, and whether influenza viruses may vary in antigenic characters under different conditions and at different times. Andrewes has postulated a basic virus of low virulence to man and experimental animals which under certain conditions may evolve by mutation into types of greater antigenic complexity and higher virulence—such as the types A and B, which can be more or less readily isolated by virtue of their pathogenicity to animals. Only continued investigation in the course of time will settle these questions.

The virus has been successfully cultivated in the laboratory in chick embryo tissue cultures and in the chorio-allantoic membrane of the chick embryo in the egg. The lesions produced by the growing virus in the embryo are insignificant at first, but passage increases virulence. The virus also flourishes in the allantoic sac, and the fluid from the sac is a useful source of virus-containing material for experimental studies. Burnet has used the method of inoculation into the amniotic cavity of the chick embryo and has studied in this way the pathological changes produced in the embryo (see Beveridge and Burnet). He has pointed out that the tracheal fluid presents

characteristic appearances, becoming turbid and containing vacuolated phagocytic cells with eosinophilic granular material in the vacuoles. The multiplication of the virus in the amniotic cavity can be demonstrated for diagnostic purposes by testing the fluid for its agglutinating properties towards fowls' red corpuscles.

As shown by Hirst, and independently by McClelland and Hare, material containing the influenza virus, e.g. chick embryo cultures, possesses the property of agglutinating the erythrocytes of the fowl and this reaction is inhibited by specific antisera, without cross-reactions between the A and B viruses. The degree of the agglutination reaction appears to depend on the concentration of virus in the test material and even when the virus is inactivated by heat there may be no reduction in the titre of the reaction. The erythrocytes of various birds, reptiles, and amphibia are susceptible to agglutination by virus-material, and also those of man and various rodents (see Clark and Nagler). The reaction with fowls' erythrocytes and its inhibition by specific antisera has been utilised for assay of antibodies, e.g. in sera of cases, convalescents, and immunised persons and animals, and results obtained by this method have in general been parallel with those of the virus-neutralisation test (*vide supra*). It should be noted that this haemagglutination reaction is also produced by certain other viruses, e.g. vaccinia, herpes.

The haemagglutinin is removed by absorption with fowls' cells, and later the virus can be eluted from these cells, which have then become inagglutinable by the virus. Working with a number of viruses possessing haemagglutinating properties, Burnet and his associates have shown that red cells treated with a particular virus, and thus rendered resistant to further agglutination by that virus, remain agglutinable by certain other viruses. These haemagglutinating viruses, therefore, can be arranged in a series such that red cells made resistant to one of them are inagglutinable by those before it in the series but not by those after it. Thus, for example, red cells treated with a normally inactive virus are rendered resistant to the virus of mumps, Newcastle disease, and influenza.

It has been demonstrated by treatment with the products of various bacteria that the action of viruses and bacterial products on the surface of red cells is a function of enzymes, and that such virus-enzymes may play a part in the invasion of tissue cells by the virus. In further studies of the enzyme action of viruses on cells, Burnet (1948) and his associates have shown that an enzyme derived from cultures of the cholera vibrio alters the surfaces of red cells in the same way as certain viruses. They postulate an effect on specific 'receptors' of the red cell and designate the active principle 'receptor destroying enzyme' (R.D.E.). They have also found that the influenza virus, when it enters the cavity of the tissue, renders the tissue resistant to the virus. The substrate on which the enzyme acts is responsible for the group-specificity of the red cells, and Burnet has shown that this involves a reaction between the virus and the findings are of viruses and the

special interest in their bearing on the pathogenesis of susceptibility or resistance of the tissues to them. The possibility of creating resistance artificially by means of a 'receptor destroying enzyme', e.g. of bacterial origin, must await further investigation.

Due to the variability of the influenza virus, and studies of the virus in two phases in the embryo, isolated allantoic fluid, and a higher order of the virus, the findings are of viruses and the strain changes, a new derivative phase is formed. In the transformation there are unstable intermediate forms in the amniotic sac, and in the cells of the

other birds and mammals referred to above. The D phase is also the more lethal to the chick embryo. Thus influenza A virus as propagated in eggs, e.g. for vaccine production, represents a different phase from that of the natural virus. According to Hirst (1947), the O→D transformation is not associated with a loss in the potentialities of the virus, but depends mainly on an enhanced hæmagglutinating action towards fowls' erythrocytes. The B virus does not apparently show any well-defined O→D change.

Another type of variation has been recorded by Burnet—the acquisition by certain strains, on repeated passage in the chorio-allantois, of highly virulent endotheliotropic characters, so that they produce marked hæmorrhagic lesions and are lethal to the embryo. Such findings emphasise the lability of the virus and are possibly significant in relation to the changes which the virus may undergo when multiplying in its natural host and spreading rapidly from person to person. The genesis of the great pandemics of influenza remains obscure, though it may be reasonably supposed that they have been caused by types or strains of the virus of greatly exalted virulence and infectivity, and possibly different biological characters, as compared with the types isolated in recent years.

Immunity. As already mentioned, ferrets after recovering from the experimental disease are immune to reinfection, i.e. by nasal inoculation. This immunity lasts for about three months, and is associated with the presence of type-specific neutralising antibodies in the serum, but after resistance to nasal inoculation has declined antibodies may still be present in the blood. Moreover, animals are insusceptible to infection by other routes, but if so inoculated develop antibodies and yet are still susceptible to nasal inoculation. However, previously immune ferrets in which immunity has declined, may be again rendered fully resistant to nasal inoculation by subcutaneous injection of virus. It has also been found that though subcutaneous injection of live virus or virus inactivated by formalin may not render ferrets or mice fully resistant to nasal inoculation, there is less likelihood of resultant pulmonary lesions. Thus, artificial immunisation of animals has so far produced only a limited degree of immunity and it is difficult to attribute the solid immunity following recovery from infection to serum antibodies.

It has been recognised that in the human subject immunity following recovery is not permanent. Francis and his associates have recorded the occurrence of two attacks of the disease within four months following nasal inoculation of the same strain of the B virus. Specific antibodies in the serum of cases and convalescents have been demonstrated by the neutralisation test and by the inhibition of agglutination of fowls' red corpuscles (*vide supra*). The presence of antibodies in healthy persons has been generally accepted as an index of previous infection.

Extensive studies have been carried out with the object of attaining a means of practical immunisation against the epidemic disease and various forms of vaccines have been used. For this purpose concentrated virus cultures from the allantoic sac of the embryo chick have been most used, the virus being inactivated by formalin. Such vaccines when injected subcutaneously bring about the appearance of specific antibodies in the blood. Some observers have recorded promising results in the prevention of the disease by vaccination prior to an outbreak (Hirst *et al.*, Francis *et al.*), but no definite statement can yet be made as to the practical efficacy of the method. Hirst and his colleagues have concluded from their observations that a definite degree of protection is produced, that it persists for a year and that the maximum protection occurs within a few weeks of vaccination. They recommend vaccination on the threat of an epidemic.

Antisera with high antibody content have been produced in the horse and it has been found that such serum increases the resistance of mice to nasal inoculation of virus. The possibility of passive immunisation as a therapeutic measure in the human disease would merit further study.

In regard to the problem of immunity, it is of interest that a virus-inactivating principle has been demonstrated in the nasal secretion of normal persons; it inactivates all types of the influenza virus and also certain other viruses, e.g. herpes. This property of the secretion is destroyed at 100° C. The nature of the protective agent is still obscure, though it has been suggested that it can be identified with serum antibodies which, of course, may be present also in normal persons as a result of previous infection.

Laboratory Diagnosis. For this purpose, in the acute stage, nasal or throat washings or sputum are instilled into the nose of a ferret, the presence of virus being indicated by the characteristic signs and symptoms (*vide supra*); moreover the infection may be passed from these animals to others and if the animals are inoculated under ether anaesthesia pneumonia may be produced.

After the patient has gargled his throat with 10 to 15 c.c. of saline, this is mixed with 5 c.c. of broth in a container. The latter is then packed in ice and sent to the laboratory for intranasal inoculation of a ferret as soon as possible.

Burnet has recommended as an alternative the method of 'amniotic inoculation' the results of which have already been described. The inoculum consists of 0.25 c.c. of throat washings containing 25 per cent serum broth in saline, and lightly centrifuged (together with 25 units of penicillin and 0.1 c.c. 5 per cent. solution of sodium sulphamerazine, in order to suppress any pathogenic bacteria which may be present); to increase the chance of a positive result, 5 eggs or more should be inoculated with each specimen. A diagnosis of infection may also be made about two weeks after the onset of the illness by demonstrating specific antibodies in the patient's serum, the neutralisation test being used or the inhibition of agglutination of fowls' erythrocytes. (It is desirable, by way of control, to test also a specimen taken as early as possible after the onset.) The complement-fixation reaction may also be applied for the same purpose. If appropriate strains of the A and B virus are available the type of infection may be recognised by means of the neutralisation test.

Influenza-like Diseases of Domestic Animals. Reference has already been made to swine influenza and the virus of this condition. Shope has made the remarkable discovery that pigs acquire the virus by feeding on earth worms. The latter harbour larvæ of the lung worms of the pig, these larvæ under natural conditions may contain the virus and convey it in 'masked,' (non-infective) form to the mammalian host. The virus becomes activated in the pig, e.g. experimentally by giving repeated intramuscular injections of *B. influenza suis*; and activation appears to occur naturally when climatic conditions are adverse, as in autumn. Influenza-like conditions have been recognised also in other animals and by analogy these are probably all of virus origin. Influenza of the horse belongs to this group and there is some evidence that a virus is responsible, though the disease requires further investigation. Recently special attention has been paid to 'influenzal pneumonia' of calves, which is a very prevalent condition and, besides the pneumonic lesion, is characterised by an acute enteritis often referred to as 'scours'. A filterable virus has been demonstrated in the lungs and transmitted successfully to mice: moreover, from the latter animals, calves have been experimentally infected (see Baker).

INFECTIOUS CORYZA ('COMMON COLD')

The infectious nature of this prevalent condition is now well recognised, and the infection can frequently be traced to contact with other cases of the same condition, the incubation period being within three to four days. The condition is characterised by a preliminary dryness and congestion of the nasal mucosa, often with sneezing, followed by an abundant watery discharge from the nostrils; there may be some degree of malaise, headache, and, in some cases, slight fever. After two or three days the discharge becomes less,

and tends to assume a purulent character; restoration to normal then occurs after a varying number of days. It is to be noted that in the purulent stage secondary spread may occur, e.g. to the nasal sinuses, middle ear, and at this stage various pyogenic organisms commonly found in inflammatory conditions of the upper respiratory passages may be present, e.g. staphylococci, streptococci, pneumococcus, *B. influenza*, *D. catarrhalis*, etc.

In 1914, Kruse showed that the condition was due primarily to a filterable virus, and he was able to reproduce the condition in volunteers after an incubation period of one to four days, by introducing into the nose the filtered nasal secretion from a case. The filtrate, obtained by means of a Berkefeld filter, was apparently free from any recognisable organism. Foster claimed to have confirmed these results. Ohtsky and McCartney, using as the inoculum naso-pharyngeal secretions from early cases in the first three to eighteen hours of the disease, the material being filtered through Berkefeld V and N filters, succeeded in transmitting the condition to volunteers, and further, were able to pass it from an experimentally infected case. Intratracheal injection in rabbits both with filtered and unfiltered naso-pharyngeal washings was without effect. These observers were unable to isolate by culture in the Smith-Noguchi medium, and on anaerobic blood-agar plates, any specific cultivable organism.

Shibley, Mills and Dochez, having observed that young chimpanzees readily develop nasal catarrh on contact with affected persons, were able to reproduce the condition in a large proportion of these animals inoculated by instilling into each nostril fresh, filtered nasal washings from human cases at the acute stage. The incubation period was thirty-six to forty-eight hours. The filtrates after heating were ineffective, as were also unheated filtrates from nasal washings of normal persons obtained at a time when coryza was not prevalent. On several occasions the condition was transmitted in series to further animals, also others contracted catarrh by contact with those affected. An important observation was that coincidently with the appearance of symptoms, pathogenic types of organisms such as the pneumococcus, *Streptococcus pyogenes*, and Pfeiffer's bacillus increased greatly in the naso-pharynx, and they appeared in the nose, where they seldom occur normally in apes. Immunity was found to last three to four months. During an influenza epidemic recovered animals, which were in the refractory period, contracted a more severe condition, as regards constitutional and respiratory symptoms, from contact with a keeper. This suggested that influenzal infection is distinct from that of the common cold. Dochez, Mills and Kneeland succeeded in cultivating *in vitro* the virus of the common cold, by a method previously applied in the cultivation of certain other filterable viruses. They used a medium consisting of a buffered broth containing an emulsion of chick-embryo tissue and with 1-2,000 cysteine added to yield a suitable reduction potential. They were able to reproduce colds in volunteers by inoculation with the cultivated virus even after fifteen subcultures. It has been possible to cultivate the virus artificially for long periods, up to one year.

Recently, further researches on the transmission of the common cold to human volunteers have been carried out in America by several groups of investigators, and in Great Britain by the Common Cold Research Unit of the Medical Research Council. Their findings broadly confirm earlier observations on the transmissibility of the condition and the virus nature of the infective agent. Virus strains have been cultivated by the American workers in the fertile egg, e.g. in the allantoic sac, and it has been shown that a cultivated virus when inoculated into the human subject reproduces the disease. Some evidence has also been obtained (Dingle *et al.*) of the existence of different forms of infection which would be classified as 'colds'. Thus, one form studied was a severe febrile catarrh with sore throat, this condition was transmitted experimentally

(though in somewhat milder form) after an incubation period of 5 to 6 days. Another type took the form of a severe coryza with fever, and was transmitted after an incubation period of 24 to 48 hours. Convalescents from the former were immune to reinoculation with material from this infection but not to inoculation with virus from the latter, while convalescents from the latter type were susceptible to reinoculation with material from the same condition even 19 days following recovery—the post-infection immunity being apparently very transitory. The question thus arises of the existence of different types of virus causing simple catarrhal conditions of the upper respiratory tract, which would fall under the clinical designation of the common cold. However, the full significance of these recent observations and the character of the viruses involved await further research. (See Pollard and Caplovitz; Dingle *et al.*, Topping and Atlas; Common Cold Research Unit)

Mixed vaccines prepared from the common organisms found in coryza (*vide supra*) have been extensively used with a view to prophylaxis. Carefully controlled tests of the efficacy of these vaccines have been made on a statistical basis (Jordan and Sharp; Ferguson, Davey and Topley), but the results have not established any definite prophylactic properties.

From these observations it would appear that the common infectious 'cold' is primarily due to a transmissible filterable virus, which may predispose to secondary infection with pyogenic bacteria.

VIRUS PNEUMONIA

The occurrence of pneumonia in which the ordinary causal bacteria cannot be demonstrated in the sputum or lung tissue appears to be increasing, especially in the United States of America. The viruses of influenza and the psittacosis group, as well as the rickettsia of Q fever (*q.v.*), may all produce pneumonic lesions in man; but there is evidence that other viruses may also be responsible. Such cases have been designated 'primary atypical pneumonia'. The illness is usually of gradual onset and terminates by lysis, but although death is rare, there is a long period of disability. The essential pathological change is an interstitial pneumonia, the cells in the infiltration being mononuclears. In some cases a virus has been demonstrated which is weakly virulent for cotton rats (*Sigmodon hispidus* and *eremicus*), hamsters, etc., on intranasal inoculation with sputum or lung suspension, lung lesions resulting (Eaton *et al.*), this virus passed through the coarser filters. Attempts to establish the infection by serial passages in animals have given irregular results. More success was obtained by inoculating the amniotic sac of chick embryos and repeated passages were obtained in this way, infectivity for animals being retained. No cell-inclusions or elementary bodies were demonstrated. The passage virus produced immunity to infection with material derived directly from the human disease. Also, serum from recovered human cases neutralised the pathogenic action of the passage virus for cotton rats, while serum taken at the acute stage of the disease was inactive in this respect. Accordingly, the evidence as a whole supports the conclusion that one is dealing with a virus derived from the pneumonic patient. An important source of fallacy in such work has been the frequent occurrence in the respiratory tract of normal rodents of latent pneumotropic viruses which became exalted in virulence by repeated passages until they caused pneumonia (see Horsfall and Hahn). The latter were not neutralised by the serum of human convalescents.

A number of cases of pneumonia have been considered also as of virus origin, since other causes were excluded (Dingle *et al.*; Drew *et al.*). A feature

in the cold (Peterson *et al.*; Turner *et al.*) Apart from trypanosomiasis, marked cold agglutinins are not met with in other conditions

Method of demonstrating cold agglutinins To 0.3 c.c. of doubling dilutions of the serum in a series of tubes 0.1 c.c. of a 3 per cent suspension in normal saline of the previously washed red cells is added and the mixtures are kept overnight at 5° C. Agglutination with a serum dilution of 1 in 128 or higher, which disappears on warming at 37° C., is highly significant, since sera from other diseases seldom yield a titre of over 1 in 16. As a rule the reaction begins to be positive one to two weeks after onset of the illness, reaches its height at the end of the third week, then declines quickly

MEASLES (MORBILLI)

The earliest reliable experimental observations on the etiology of this disease were made by Hektoen in 1905, who reproduced measles in volunteers by the subcutaneous injection of blood from cases at an early stage of the illness. No bacteria could be demonstrated in the blood by culture. Anderson and Goldberger, a few years later, were successful in transmitting the

secretions of inoculated animals. Though some workers have been unable to confirm these results, it seems now well established that a virus is present in the blood at an early stage, which is experimentally transmissible to monkeys, reproducing a condition similar to human measles. Blake and Trask inoculated monkeys (*Macacus rhesus*) intratracheally with filtered naso-pharyngeal washings from cases at the commencement of the eruptive stage, and produced a condition presenting many similarities to human measles, with lesions of the skin and the mucous membrane of the mouth corresponding histologically to the measles series of animals attack. Shaffer

naso-pharyngeal washings from early cases produces typical infections in children and monkeys, the disease in the latter being milder than the usual human infection. They have cultivated the virus from naso-pharyngeal washings in the chorio-allantoic membrane of the chick embryo and reproduced the typical disease in children and monkeys by injecting material obtained after several subcultures. Taniguchi and his associates claimed to have established in rabbits several strains of virus from the blood and filtered

of measles were produced. Also, the serum of convalescent patients had a neutralising action on the virus. Other workers have failed to transmit the disease to rabbits and it is doubtful whether animals other than monkeys are susceptible (see Gordon and Knighton, Hurst and Cooke).

There is now a considerable amount of evidence that the measles virus can be cultivated in the chorio-allantoic membrane of the embryo chick and in tissue cultures, and that the cultivated virus under experimental conditions can reproduce the disease, though repeated cultivation may lead to attenuation (see Rake, Mayer, Stokes *et al.*). Knowledge is still lacking regarding the virus bodies, though cell inclusions have been described in the nasal epithelium and in the Koplik lesions of the mucous membrane of the mouth (Broadhurst *et al.*). The relationship of these to the virus requires further study.

The immunity following an attack of measles has long been recognised,

and experimental work has shown that it depends mainly on specific properties resident in the blood serum (Nicolle and Conseil). The serum of convalescents, taken one week after defervescence, has been used both in the prophylaxis and treatment of measles. It has been found that if the immune serum is injected during the first five days after infection, an effective passive immunity is produced which may persist for a month. According to Brincker the appropriate dose for a child is double the number of c.c. corresponding to the age in years, injected intramuscularly. Further, an injection even after the fifth day, *i.e.* on the seventh to tenth day, tends to modify the subsequent attack, and this procedure may be adopted with advantage, as was suggested by

for the supply of convalescents' serum, centres have been established in various countries for collecting and distributing the serum, which is obtained from convalescent donors over ten years of age (it is necessary, of course, to ensure that they are free from any other systemic infection, *e.g.* syphilis, tuberculosis). Pooled serum is used, but at present there is no means of standardising the serum. When convalescent serum is not available pooled normal adults' serum in a dose double that of convalescent serum can be used. Adult serum is mainly applicable to children under five years; above this age inconveniently large amounts are required. Preparations of globulins extracted from human placentas contain the measles antibody and can likewise be used for prophylactic purposes, the dose of such preparations being generally 2 c.c. injected intramuscularly (McKhann and Chu). It is stated that the efficacy of placental globulin is intermediate between that of convalescent and 'adult' serum. Recently preparations of the gamma globulin of human serum have been shown to possess marked prophylactic properties; a dose of 2 c.c. given prior to the ninth day after exposure to infection completely prevents the disease in children under four years of age (Greenberg *et al*). The production of a mild form of measles by inoculation with a cultivated virus (*vide supra*) has been suggested as a means of active immunisation.

RUBELLA (GERMAN MEASLES)

It has been generally assumed that this disease is due to a specific virus. Recently Habel claimed to have transmitted the disease to monkeys by inoculation with blood and nasal washings from cases. After an incubation period a slight febrile illness developed with leucopenia, relative lymphocytosis and an eruption similar to that of the human disease. Confirmatory evidence is required.

DURAND'S DISEASE

The virus was isolated by intraperitoneal inoculation of a guinea-pig with blood from a patient during a short influenza-like febrile illness. In the human disease the cerebro-spinal fluid may show an increase of cells and a decrease of protein. The guinea-pig is the most susceptible to the disease. The virus is transmitted intranasally. Inoculation with suspensions of spleen taken during the illness is followed by a febrile illness and enlargement of the related lymph glands due to hyperplasia of the reticulo-endothelial cells and there is sometimes subcutaneous oedema. About 10 per cent of infected animals die, the

spleen shows considerable enlargement and there may be pneumonic lesions. Animals which survive the infection become solidly immune to reinoculation. Subcutaneous or intraperitoneal inoculation of various other animals, e.g. monkeys, cats, dogs, rats, and mice, leads to an almost inapparent infection. But nervous symptoms (paresis, etc.) follow intracerebral inoculation of the larger animals. The virus proliferates in a mixture of 10 per cent. heated human serum in Tyrode's solution to which a small amount of minced chick embryo has been added, and it survives long after the tissue cells have died. Cultures have also been got by inoculation into the yolk sac or on to the chorio-allantoic membrane of hens' eggs. There is no cross-immunity with the virus of lymphogranuloma inguinale or lymphocytic choriomeningitis. Although the virus of Durand's disease resembles certain viruses found in the guinea-pig, the former does not spread by contact among these animals.

INFECTIVE MONONUCLEOSIS (GLANDULAR FEVER)

This condition is characterised by fever along with enlargement of lymphatic glands, especially in the neck, and there may also be splenomegaly. Non-granular leucocytes are increased in the blood, cell counts of over 30,000 per c mm. being recorded. Abnormal cells are a feature, these resembling

several relapses. In infective mononucleosis marked agglutinins for sheep red corpuscles (heterophile antibody) appear in the blood and this may be of value in diagnosis (the Paul-Bunnell test), agglutination with the patient's serum in a dilution of 1:256 or higher may be regarded as positive (see Dempster). A positive reaction may not develop until late in the disease,

the ox, but not by a suspension of guinea-pig's kidney, whereas the agglutinins developed in serum disease are not absorbed by ox red cells, but are reduced by contact with guinea-pig's kidney (Dempster, see Barrett for methods of procedure). In many cases of infective mononucleosis no organisms of any kind have been demonstrated in the blood or lymph glands. Wising observed the development of the condition in a volunteer following transfusion of such blood from a patient. But apparently a similar clinical picture may be caused by infection with *Bact. monocytogenes* (see Webb) and *Toxoplasma* (Bland).

MUMPS (INFECTIVE PAROTITIS)

It has been established by the work of Granata and others that rabbits, monkeys, and cats develop lesions when inoculated with sterile filtrates of the saliva from cases of mumps or of fluid obtained by puncturing the parotid gland. The disease can be transferred in series through cats by means of inoculation into the parotid gland or testis (Wollstein). Swelling and tenderness of the inoculated organs develop after five to nine days, and the microscopic changes are similar to those in the human disease. The virus disappears from the saliva between the sixth and ninth day of the disease, it is present in the blood in severe cases. The disease can also be produced in monkeys by introducing infective filtrates into Stenson's duct, and a series of passages

have been effected in this way (Findlay and Clarke). Further, it has been shown that the virus can produce mumps in susceptible persons after passage in monkeys (Johnson and Goodpasture). Blood serum from a recovered animal when mixed with tissue containing the virus neutralises the action of the latter. After intracerebral inoculation of filtered mouth washings from acute cases of mumps into monkeys, meningitis and degenerative changes in the nerve cells of the brain and cord occur, and there are changes in the parotid when survival is sufficiently prolonged (Gordon). Meningo-encephalitis, usually of a mild type, may also occur as a clinical complication of mumps. The virus survives in 50 per cent. glycerol for at least five weeks and is resistant to drying and freezing. It has been cultivated in the fertile egg, e.g. in the allantoic sac and the amniotic cavity (see Burnet, 1946).

Complement-fixing antibody is present in the serum during the illness and in convalescence, and the complement-fixation reaction has been used for diagnostic purposes, e.g. in mumps encephalitis (Kane and Enders). Infected monkey parotid gland or chick embryo fluids in which the virus has been cultivated may be used as antigen for the serological test. As in measles, convalescent serum has been used with a certain amount of success in the prophylaxis of mumps, the serum being injected within five days after exposure to infection.

The mumps virus, cultivated in the amniotic cavity of the chick embryo, possesses hæmagglutinating properties like those of the influenza virus, and the serum of convalescents specifically inhibits this reaction. The red cells of the fowl, pigeon, guinea-pig, and man have been found susceptible to such agglutination. The hæmagglutinating property is removed by absorption with fowls' cells, and the virus can later be eluted from the cells, which have now become magglutinable by the virus (see Beveridge and Lind).

EPIDEMIC KERATO-CONJUNCTIVITIS

An eye condition, tending to occur in epidemics and appearing especially on the sea-coast ('ship-yard conjunctivitis'), has been recognised as a clinical entity in Europe, North America, and the Middle East. It is characterised by acute onset of moderately severe conjunctivitis with painful enlargement of the pre-auricular gland and often swelling of the submaxillary gland. The conjunctival secretion shows fine fibrinous threads and a few monocytes, no inclusion bodies are found in epithelial scrapings. One eye may be affected first, the second becoming involved later. Macular keratitis or other corneal changes are usually present also; these lesions tend to be very persistent. In some cases in Egypt the association of this condition with skin lesions (especially seborrhœic dermatitis) of the scalp or face has been noted, usually on the same side when one eye was affected (O'Donovan and Michaelson). Sanders *et al.* isolated from conjunctival scrapings a filterable virus by means of tissue cultures grown at room temperature, it caused death of mice and rabbits on intracerebral inoculation and the disease could be passed through mice in series. The virus was neutralised by the serum of convalescent patients with herpes or lymphocytic choriomeningitis, but obtained tissue cultures of the rabbit's cornea.

EPIDEMIC GASTRO-ENTERITIS

Recently experimental evidence has been obtained by Hodes and Hodes investigated four epidemics in new-born children in two hospitals in the Baltimore-Washington area. A large proportion of the infants were attacked and in

three outbreaks there was a high mortality, no bacteria of specific intestinal pathogenic types were found. The presence of a filterable agent was demonstrated in the stools from cases in each outbreak by nasal feeding of the material to young calves averaging one to three weeks old. Diarrhoea with the presence of mucus and blood developed after an incubation period of two to five days, and the intestines showed hyperæmia with swelling of the lymphoid tissue and mesenteric lymph glands. Passage was effected with filtered fæces from the calves, also spread of the infection by contact occurred. Neutralising properties were demonstrated in the serum of recovered babies and in a rabbit injected with the infective material. For inactivation the virus required to be exposed to above 70° C for one hour. It was not found in a number of healthy babies or calves, and it differed apparently from the causal agent of a natural case of calf scour; also it was not identical with Baker's virus of pneumo-enteritis of calves, which is transmissible to mice. Gordon *et al* investigated an outbreak of gastro-enteritis among adult inmates of a New York State hospital and showed that filtrates of the fæces of early cases when administered by mouth to volunteers reproduced the disease after an incubation period averaging three days. The material was inactivated by autoclaving at 121° C for twenty minutes. Several passages were effected with filtered material from experimental cases. Recovery from an attack was followed by immunity to reinoculation. Both the viruses from infants and adults remained active when kept at -70° C. It remains to be determined whether such findings apply generally to epidemic gastro-enteritis in other localities.

CANINE DISTEMPER

This infectious disease, which is common in dogs, foxes, fitches, and ferrets, has been proved to be of virus origin, though secondary bacterial infection contributes often to the complete pathological condition. The virus infection is generalised from the outset, but there is a pronounced localisation in the respiratory system with resulting catarrhal inflammation, and pneumonia may ensue. The gastro-intestinal tract may also be affected and in some cases encephalitis occurs. It is possible that in certain of the encephalitic cases, an independent virus is responsible for the effects on the central nervous system (Verlinde). The disease can be reproduced experimentally in dogs and ferrets by subcutaneous injection of blood, tissues, *e.g.* spleen, and discharges from the natural disease.

Little is yet known about the characters of the virus. Both cytoplasmic and intranuclear inclusions have been described in the epithelial cells of the respiratory passages, urinary bladder, and certain other tissues, and their occurrence in the nose and bladder has been considered by Green and Evans to be diagnostic. Cultivation of the virus in the chorio-allantoic membrane of the embryo chick has been achieved. Prophylactic vaccination has met with considerable success. The usual vaccine is a formolised emulsion of spleen and mesenteric glands from infected dogs, and injection of this preparation is followed after fourteen days by an injection of active virus from the spleen and glands of infected ferrets. Another method of immunisation is to inject virulent ferret virus followed after one and a half hours by a dose of immune serum produced in dogs. This immune serum has also been applied for therapeutic purposes (see Laidlaw and Dunkin, Dalling).

It should be noted that '*Cat distemper*' is due to an entirely different virus, more related to the psittacosis virus and the virus of pneumonia in mice described by Nigg (*vide p.* 582). *Feline infectious enteritis* has also been found to be of virus origin but distinct from the cat distemper virus (see Hammon and Enders).

RINDERPEST

This is an infectious disease of cattle and buffaloes occurring mainly in Asia and Africa. It is characterised by signs and symptoms of a generalised infection with a haemorrhagic inflammation and ulcerative condition of the

alimentary tract. A filterable infective agent can be demonstrated in the blood, tissues, and discharges, by experimental transmission of the disease to cattle and goats, though passage in the latter species leads to attenuation of the virus for cattle. It has been shown that in the blood the virus is associated with the leucocytes and is not free in the serum. The virus is sensitive to drying and does not resist glycerol. So far it has not been cultivated. The disease is transmitted by the nasal and other discharges and the virus probably invades the body by a mucous membrane. Recovery is associated with a durable immunity. Large-scale immunisation has been successfully practised by the use of a vaccine consisting of formalised spleen pulp from infected animals, along with immune serum. A virus adapted to goats (*vide supra*) has also been employed with successful results, the dried spleen tissue being used as the vaccine. (See Daubney; Minett; Pfaff; and Schein.)

SWINE FEVER

This is a highly infectious disease occurring in pigs, but not transmissible naturally to other domestic animals, man, or laboratory animals. It takes the form of a general infection with a secondary or associated hæmorrhagic inflammatory condition of the intestinal tract, and in chronic or protracted cases necrotic lesions in the bowel. The etiological problem of the disease has been complicated in the past by the occurrence of *B.* (or *Salmonella*) *sniffestifer* in the intestinal lesions, but this infection must be regarded now as secondary. In some cases there may be secondary pulmonary lesions in which a *Pasteurella* group organism is found also, representing a secondary infection and this has led to confusion of the disease with swine plague. It must be recognised that *Salmonella* and *Pasteurella* organisms are only found in a proportion of cases.

It has been known for some time that a filterable virus is primarily responsible for swine fever, filtrates from blood, serum, bile, urine, and various tissues of infected pigs reproduce on inoculation in pigs the typical disease, and once established in this way the virus can be transmitted through a series of animals by passage experiments. Recovery from the natural and experimental disease is associated with the formation of immune serum of immune animals has animals remain carriers of the infection. Presumably natural immunity is by ingestion.

Our knowledge of the virus is still incomplete. It has been found to possess considerable resistance to physical and chemical agents. It survives in dried material and in preserved meats. It is resistant to 50 per cent. glycerol. It has been grown in the chorio-allantoic membrane of the embryo chick and in tissue cultures from pigs' testes.

Much work has been done on prophylactic immunisation. Infected blood along with an immune serum has been used for the purpose. Recently the 'crystal violet vaccine' has been successfully employed. The virus can be rendered inactive by this dye while retaining its immunising action: 0.05 per cent. crystal violet is added to infected blood along with disodium phosphate, and the mixture is kept at 37° C. for fourteen days before use. (See Uhlenhuth *et al.*, Tenbroeck.)

VIRUS MYOCARDITIS

The spontaneous occurrence in various animals of acute interstitial myocarditis has been recorded. Helwig and Schmidt observed this condition

in several anthropoid apes in captivity and isolated from one of them a filterable virus by injecting pleural fluid and spleen suspension into mice. Inoculation by various routes—intravenous, intraperitoneal, subcutaneous, nasal, etc.—was successful. Paralysis developed in a few days and death occurred in seven to ten days usually. In addition to the heart lesion, encephalo-myelitis was marked *post mortem*. Passage was successful, the virus being widely present in the tissues; but after ten passages the virulence declined. Hamsters were also susceptible and then rabbits in descending degree. Rats suffered no ill effects from rich inoculation with the virus. Growth of the virus in the allantoic sac of hens' eggs was not regularly successful. Recovered mice showed neutralising antibodies in their serum, and Smadel found that serum from soldiers convalescent after 'three day fever' in Manila neutralised the virus, whereas serum from cases of rheumatic fever failed (Schmidt).

INFECTIOUS NEOPLASMS OF RABBITS

been isolated

and also filtered material and lesions develop rapidly, first at the site of inoculation and subsequently in other parts, including the spleen and lymphatic glands. The disease runs a rapid course with a fatal result usually in a week or a fortnight. Elementary bodies have been demonstrated and the size of the virus has been estimated as 125 to 225 $m\mu$. The virus has been cultivated in the chorio-allantoic membrane. Acidophile cytoplasmic inclusions have been described in epidermal cells, which according to Rivers are involved in the lesions, apart from the proliferation of connective tissue elements (See Rivers *et al*).

Infectious fibromatosis was first described by Shope in cotton-tail rabbits. The disease is non-fatal and spontaneously retrogressive. Filtrates of the tumours reproduce the lesions. The size of the virus is apparently about 125 to 175 $m\mu$. Cytoplasmic inclusions may be found in the lesions. It is of interest that a variant strain has been obtained from the fibromatosis virus, which produces an inflammatory condition without neoplastic changes, and it is of further interest that, as shown by Shope, animals recovered from the fibroma virus are immune to myxomatosis, the converse cross-immunity has also been observed. Moreover if a mixture of heated (60° to 80° C.) inactive myxoma virus, and the fibroma virus are inoculated, myxomatosis results. The explanation of this finding is still doubtful, though it seems clear that the two viruses have a definite relationship to one another (See Shope, Andrewes, Berry and Dedrick.)

Infectious papillomatosis also occurs in cotton-tail rabbits and takes the form of multiple skin papillomata. The disease is transmissible in these rabbits and is retrogressive as a rule. When, however, domestic rabbits are inoculated with infective material, some of the resulting papillomata become malignant. Papillomatosis so produced in the domestic rabbit is not readily transmissible, and a filterable virus is difficult to demonstrate. The virus is much smaller than those of the other rabbit tumours, from 23 to 50 $m\mu$. It can resist heating up to 67° C for thirty minutes. There is no evidence so far that the presence of the virus is essential for the propagation of the derived malignant tumour when inoculated into other rabbits (See Sharp *et al*, Shope, Shope and Hurst).

VIRUS DISEASES OF DOMESTIC BIRDS

Several infectious diseases of domestic birds are due to viruses, including *fowlpox*, which has already been considered among the pox diseases of man and animals. One of the most infective of these is *fowl-plague*; thus an exceedingly minute quantity of blood, 0.0000001 c.c., from an infected bird serves as an inoculum in the experimental transmission of the disease to chickens. The virus is also present in nasal and cloacal secretion. Filtrates are likewise infective and the size of the virus is estimated as 60 to 90 m μ in diametral size. It is of special interest that the virus in the blood has been found to be concentrated in the leucocyte fraction after centrifuging. The virus can be cultivated in the chorio-allantoic membrane of the embryo chick.

The so-called *Newcastle disease* of fowls was reported first by Doyle. It bears some resemblance to fowl-plague. It is transmissible experimentally to fowls and pigeons, whereas the fowl-plague virus does not infect the latter. A virus has been demonstrated and its size estimated at 80 to 120 m μ . Cultures are obtained in the chorio-allantoic membrane and cause marked lesions in the embryo. The allantoic fluid yields the agglutination reaction with fowls' erythrocytes as described by Hirst in the case of influenza virus cultures. Moreover, this virus when inoculated in mice intranasally produces a pneumonic lesion. It has no immunological relationship, however, to the influenza virus.

Other infectious diseases of fowls due to viruses are *infectious laryngotracheitis*, *infectious bronchitis*, *blue comb*, and *leukemia*. Though a virus has not yet been proved in *fowl-paralysis*, the evidence available at present points to a virus causation of the disease.

Of special interest in regard to the whole problem of the etiology of neoplasms is the virus-like agent which has been demonstrated in the *Rous sarcoma*. This tumour can be transmitted to fowls by the inoculation of filtrates from the tumour, and the active agent is generally regarded as a virus similar to those of true infectious diseases. Other 'filterable tumours' have also been described in fowls and other birds. Elementary bodies have been demonstrated in filtrates of the Rous sarcoma, and the size of the virus particles is estimated with gradocol filters as 75 to 150 m μ . According to Gye the power of the virus to produce a tumour depends on an additional factor and he also claims to have cultivated the virus anaerobically in a medium containing broth, rabbit's serum, and a fragment of chick embryo.

CHAPTER XXXI

BACTERIOPHAGE

It has long been known that bacteria in culture are apt to undergo a process of autolysis, becoming disintegrated and sometimes disappearing, such changes set in earlier and are more pronounced in some species than in others. However, it has come to be recognised that rapid lysis may be set up by some special agents or stimuli, and that such a lytic process is transmissible to other cultures by the products of the lysis. Twort (1915), in working with certain micrococci obtained from vaccine lymph, observed the appearance of glassy and transparent patches in cultures on agar, which increased at the expense of the ordinary bacterial growth. He found that a similar lytic change could be transmitted to fresh growths by inoculation from a glassy patch, and that in this way the change could be continued for an indefinite period of time. Further, he showed that the lytic agent was present in bacterium-free filtrates which had been passed through very fine porcelain filters. He discussed the various possibilities in explanation of the phenomenon, including that of its being due to an ultra-microscopic virus; but considered that a definite conclusion was not warranted. A similar phenomenon was described somewhat later by d'Herelle (1917), first in the case of a dysentery bacillus, and was made the subject of a long series of important researches. He formed the opinion that the lysis was due to an ultra-microscopic virus and, in fact, represented the result of a parasitic infection of the bacteria by the virus. He applied the term 'bacteriophage' to the supposed virus, and this term has come into common use; it is often used in the abbreviated form of 'phage'. Various phages have now been obtained which act on many different organisms, including the Gram-negative intestinal commensals and pathogens, *V. cholerae*, *B. pestis*, staphylococci, streptococci, *B. diphtheriae*, *B. subtilis* (in the vegetative stage), etc.

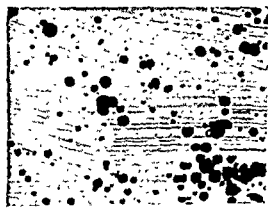


Conditions of Action. When bacteriophage is added to an agar surface culture a clear band develops along the line of inoculation, and this may involve the whole culture, the surface of the medium becoming clear of growth (Fig. 160). Often colonies develop on the clear surface after a time and these are formed from resistant organisms which have escaped the lytic action. When a young broth culture, turbid in appearance, is inoculated with bacteriophage, it gradually becomes clear as the lysis goes on and ultimately all trace of turbidity may disappear. But if the broth is inoculated with the organisms and at the same time a very small amount of phage is introduced, multiplication of the bacteria occurs for four or five hours—and actually is stimulated as compared with the rate of growth in the control culture lacking phage, then clearing rapidly takes place. Fluid cultures are

seldom completely sterilised by the phage; turbidity develops again later, owing to the growth of resistant organisms. If a well-developed young culture of an organism in broth be inoculated with a lytic dose of phage, and then some of it is spread over an agar plate the film of growth comes to be beset with small circular clear areas or 'plaques', which represent points of action of the lytic principle or, according to d'Herelle's view, 'colonies of the bacteriophage' (Fig. 161). When the bacterial colonies on a plate are of some size, those affected by the lytic process may be partly cleared and present a 'nibbled' appearance.

Bail showed that when a filtrate of material which naturally contains phage, such as faeces, is allowed to act on a susceptible culture in dilutions which produce discrete plaques, the latter may not all be of the same size

infra), owing to their greater diffusibility, are those which produce the larger plaques. A pure strain of phage may be obtained as follows: a fluid culture is inoculated from a plaque and is filtered after clearing has occurred; high



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dilutions of the filtrate are then plated on a culture of the same bacteria; and these procedures are repeated several times, a discrete plaque being used as the inoculum on each occasion. In characterising a phage the chief features which should be noted are the size of the plaques; the completeness of lysis in their centre and whether secondary colonies develop; the form of the margins, whether uniform or irregular, and whether the edge is sharp or shelving or there is a thickened ring of growth or a zone of partial clearing, and if so, its relative width (Sertic and Boulgakov, 1933).

When a lytic dose of bacteriophage is added to a fluid culture of susceptible organisms the first detectable occurrence is the fixation of the phage by the latter, so that it diminishes greatly in the fluid. After a period there is a sudden great increase in the bacteriophage content of the medium, which is taken to be the result of setting free of phage particles by dissolution of the organisms in which they have multiplied. It should be noted, however, that the addition of a very large dose of phage to a suspension of susceptible bacteria at room temperature may bring about their death quickly—within a few minutes—whereas lysis occurs only after some hours. The rate of clearing relative to the number of particles may be accelerated by the presence of certain substances in the medium, e.g. a trace of manganous chloride.

The amount of lytic agent present in a preparation of bacteriophage may be estimated quantitatively in several ways. (a) To a series of tubes each containing a standard quantity of a susceptible culture a fixed amount of varying dilutions of the phage is added, several tubes being inoculated with each dilution, and the mixtures are incubated. The smallest amount of the phage is determined which will cause clearing in, say, half the tubes of culture to which it has been added. (b) A number of plates of agar medium

are uniformly inoculated on the surface with a dense suspension of a young culture of the susceptible organism, when these have dried a standard amount of each of a series of decimal dilutions of the lytic filtrate is spread over the whole surface of a plate. Alternatively, the mixtures of culture and phage dilutions may be prepared first and then inoculated on to the plates. After incubation, the plate showing discrete plaques is selected and their number counted. Under optimum conditions, each plaque is regarded as representing the action of one particle of phage. (c) Another method consists in adding a suitable dilution of phage to a measured amount of a standard suspension of susceptible organisms, from the time which is required for a certain degree of clearing to occur under constant conditions the number of

filtrate were plated on agar along with the susceptible strain of bacteria. From the plaque count (visible after a few hours' incubation) the concentration of phage particles in the undiluted filtrate was estimated and sufficient was then added to a young, growing broth culture of the bacteria to give slightly less than an average of one particle per standard volume (0.02 cc). This volume of the mixture was drawn into a number of capillary pipettes which were sealed at the narrow end and incubated at 37°C. At suitably short intervals batches of the tubes were taken and the whole contents of each spread separately on an agar plate. After incubation, the number of resulting plaques in each culture represented the number of phage particles derived from one organism. It was found that for some time at the beginning no change in the number of plaques occurred and then there was a sudden marked increase. This result corresponds with that obtained by microscopic examination (*vide infra*).

As regards the changes produced in the bacteria by the action of phage when observed under the microscope by dark-ground illumination, it has been found in the case of *B. coli* that the cells swell up and then suddenly undergo disruption. The staphylococcus after preliminary swelling becomes reduced in size and density and then slowly disintegrates. With *B. megatherium* there is no preliminary enlargement and the final stage of lysis occurs slowly. A number of particles is seen to form inside the organisms and to become free when the bacterial cell disintegrates. From one *B. coli* several hundred particles (phage bodies) are liberated, but from a staphylococcus not more than four (Eisenberg-Merling). The phage particles as shown by the electron microscope (Fig. 162) have often a tadpole-like appearance (Luria *et al.*). There is evidence that the clearing brought about by bacteriophage when acting on a susceptible culture is, in part at least, due to enzymes which are incapable of multiplication. For instance, in the case of those phages which produce plaques surrounded by a halo of partial clearing, it is



FIG. 162. Electron micrograph of a staphylococcus bacteriophage, shadow cast with gold $\times 20,000$ (From the National Institute for Medical Research.)

found by subculturing that the halo is devoid of phage. It is not known, however, whether the enzyme is derived from the phage or from the bacteria themselves.

Propagation and action of the bacteriophage, so far as is known, take place only in the substance of *living and multiplying* bacteria; thus phage does not lyse organisms suspended in saline nor does it increase under these conditions. No one has been able to show any increase of it in any medium *not containing the bacteria*. On the other hand, killed bacteria may fix the phage, and so also exceptionally may living organisms which are insensitive to its action. Lysis of susceptible bacteria under the influence of phage does not occur in cultures on gelatin or agar which is too concentrated, neither does it occur in the presence of tissues, as in tissue cultures; also, the addition of serum to a preparation of phage *inhibits its action*. Phage may multiply in cultures of bacteria in synthetic media. Traces of calcium are necessary both for the lytic action and propagation of many bacteriophages, these being inhibited by the addition of sodium citrate to the medium.

The relation of bacteriophage to bacterial variation has already been mentioned (p. 25). As has been seen, this is associated with changes in the antigenic constituents of the organisms. While the action of phage on the S type leads to the appearance of R forms, the reverse may also occur, though rarely. But acquired resistance to phage may not be accompanied by any change in the antigenic characters of an organism detectable by means of agglutinating antisera (Burnet). Again, the S and R forms of a given organism differ in their susceptibility to phage action; as a rule the latter are acted on by a wider variety of phages than the former. In this respect also there is considerable parallelism between the distribution of a common somatic antigen in different bacterial species and their sensitiveness to a given phage. This has been confirmed by the finding that those *Salmonella* organisms which contain the same complex polysaccharide somatic antigen tend to be susceptible to the same strain of phage. Also such polysaccharides when extracted from *Salmonella* bacilli have the property of combining with the corresponding phage and preventing its action on the susceptible bacilli (Gough and Burnet). Phages for the typhoid bacillus have been discovered, the activity of which depends on the Vi antigen. By this means it has been shown that a number of types of this organism exist

SOURCES OF BACTERIOPHAGE

Bacteriophages have most frequently been obtained from the alimentary tract of the human subject and animals (e.g. pigs, chickens), either in a healthy or diseased condition. They have also been got from organisms cultivated from lesions in the tissues, and further, they have occasionally appeared to develop in ordinary cultures in the laboratory. The usual method of obtaining a bacteriophage is to make a culture of faeces or sewage in broth along with various organisms, then filter and test for the presence of the lytic property by inoculating a culture of the same organism with the filtrate. After a bacteriophage has been obtained, its action on a given organism can frequently be increased by passing through a series of subcultures, that is to say, by promoting active proliferation of the bacteria; a certain level of activity of the bacteriophage is then maintained by further passages. Occasionally, however, it dies out spontaneously, and this occurs especially when a minute dose of phage is added to great excess of the susceptible culture.

Bacteriophages thus obtained present considerable differences in respect to the organisms on which they exert a lytic action. A phage as originally obtained from a culture of a dysentery bacillus, for instance, has usually a certain action on members of the same group, and on some varieties of *B. coli*; as a rule it has little or no action on the organisms of other groups. It has been found, however, that when a phage is added repeatedly to cultures of certain insensitive organisms, *e.g.* by propagating it in a mixture with the susceptible bacteria, lytic properties towards the former may be developed. Further, it has been found that while lytic action for one organism is acquired, it may be lost for another.

PHENOMENA OF RESISTANCE TO BACTERIOPHAGE, AND LYSOGENIC ORGANISMS

It has already been seen that when a culture which has been cleared by phage is allowed to stand, growth usually appears in it later. The organisms which thus develop are found to be resistant to lysis by the original phage. This acquired insusceptibility may conveniently be termed 'resistance' in order to distinguish it from the 'insensitive' state of a culture to a phage with which it has come in contact for the first time. In the case of a strain of organism which is susceptible to several different phages, if resistance to each type of phage is developed in the manner described above, it is found that such resistance is specific—that is, the strain which has been rendered resistant to one type remains susceptible to the others. A culture can acquire resistance to several different phages by subjecting it to these successively. Also, when an organism has developed resistance as the result of treatment with a particular specimen of phage, it may become susceptible to other phages which were without action on it in its original state. Bail has proposed a classification of bacteriophages according to the following principle. When a resistant strain of organisms has been developed by contact with a particular phage, and it is found that as a result the organisms have also become resistant to other specimens of phage, then the latter belong to the same group as that which originated the resistance.

It is noteworthy that certain cultures, although consisting entirely of organisms which show no lysis, nevertheless yield a lytic agent for other organisms; such cultures are termed 'lysogenic'. The behaviour of resistant and

a view to

Burnet,

culture has been acted on by phage are sometimes, but by no means invariably, lysogenic, *i.e.* they continue to produce a 'symbiotic' phage which lyses the original strain. In contrast with what has been stated above, the activity of phage formed by a lysogenic strain tends to decrease when the organisms are kept under conditions promoting very active multiplication, *i.e.* rapid subculturing. In these circumstances a modified ('attenuated') phage may still be produced, which is fixed by the original organisms, although it does not lyse them, it confers protection against an active phage added afterwards. Subsequently phage production may no longer be detectable and apparently the strain ceases to produce any phage. From what has been stated above it will be seen that resistance to a phage may be produced in two ways—(a) by contact with the phage leading to a strain which no longer fixes this phage, or (b) by first allowing the organisms to fix a non-lytic variant phage and thereby keeping the lytic phage out of combination with them.

It appears that a resistant strain of organisms which develops in a culture lysed by a phage is no longer capable of fixing the latter. However, as has been seen, this resistant strain may at the same time be lysogenic and also capable of fixing the phage which it produces. Accordingly, variant phages have been developed which differ from the original both in combining affinities and lytic power; nevertheless they are identical with the original strain of phage in other characters, *viz.* the size of the plaques produced, antigenic properties, and thermo-resistance (*vide infra*).

There is so far no conclusive evidence that a phage can be developed in a culture of organisms in any way, except by inoculating them with a pre-existing phage. Where there is apparent development of phage *de novo*, it seems likely that this is to be accounted for by the conditions leading to increase of virulence of one already present in an attenuated condition, *e.g.* a lysogenic strain which has ceased to produce active phage owing to too frequent subculturing, may under circumstances less favourable to vegetative growth of the bacteria (poor media and infrequent subculture) begin to produce the active phage again.

RESISTANCE OF BACTERIOPHAGE TO PHYSICAL AND CHEMICAL AGENTS

In comparison with most unicellular organisms, bacteriophage has somewhat higher powers of resistance to various agencies. For example, a lysed culture may contain the lytic principle after several years, as is shown by inoculating from it to a fresh culture of the organism originally lysed. Faces kept in sealed tubes for over a year have been found to contain the bacteriophage, practically unchanged. Further, it may withstand drying for several months. Different specimens show variation in resistance to heat, but in some instances a temperature of over 75° C. for half an hour is necessary to ensure destruction. However, the composition of the medium influences markedly the effect of heat, and resistance is much greater in the dry state than when moist. A striking observation made in the case of a lysogenic strain of the sporing bacillus, *B. megatherium*, by den Dooren de Jong was that the property of producing phage persisted after the spores had been heated for five minutes at 100° C., whereas the phage itself was destroyed at 70° C. in the same time. Similarly, in the case of a spored culture of *B. subtilis* artificially inoculated with the corresponding phage, after the spores had been boiled and allowed to germinate the resulting culture contained phage (Cowles). But when a fresh specimen of phage has been heated short of total destruction, it is found that on standing for twenty-four hours the greater part of the activity may be recovered. D'Herelle found that phage was not destroyed after being kept in 1 in 200 corrosive sublimate or in 1 in 100 carbolic acid for three days, after a somewhat longer period destruction followed. Some phages are readily destroyed by the action of strong solutions of urea or neutral salts of quinine and a few by trypsin. A staphylococcus phage was destroyed by the photodynamic action of a 1 in 100,000 solution of methylene blue in the presence of oxygen, but when fixed to living organisms either of homologous or heterologous species, it was protected. In general, substances which act as antiseptics or which denature proteins, although they may show a higher resistance than

However, bacteriophages are specially susceptible to chemical agents. Thus by means of potassium permanganate it is possible to free a culture of organisms from admixture with a phage to which it is sensitive, on the other hand, a lysogenic culture cannot

be deprived of its capacity to produce phage by such methods (Lominski). It may also be mentioned that bacteriophage persists for a long period in a

Anti-Infective Action of Bacteriophage. It has been shown under experimental conditions that an infection can be combated by administering a phage lytic for the organisms concerned (Asheshov *et al.*; and others). In the case of mice infected intraperitoneally with a lethal dose of virulent typhoid bacilli containing Vi antigen (*vide infra*), an intravenous injection of the corresponding Vi phage within an interval of several hours protected the great majority of the animals, on the other hand, a heterologous Vi phage did not have produced by by injecting

active against the organisms. The exact mechanism of the protective action of the phage, whether in directly killing the organisms or by promoting phagocytosis or otherwise, is not clear, however. In more chronic infections, beneficial effects from administration of bacteriophage might also be due either to the production of a non-specific stimulation of the defences or to its acting as a vaccine. The latter property may be very pronounced, as in the case of a phage-lysed culture of the *Pasteurella* of hæmorrhagic septicæmia of buffaloes, which confers marked protection against the disease (d'Herelle).

The fact that phages are inhibited in their action by tissues and tissue products or serum suggests a reason for the frequently recorded failures to produce a therapeutic result by their administration. It might be expected that the greatest effect would be obtained in relatively superficial infections, and success has been recorded in cholera (*q v*). In the case of bacillary dysentery, the well-controlled investigations of Boyd and Portnoy showed neither modification of the clinical features of the disease nor any influence on excretion of the organisms following administration by mouth of a potent phage which both reached the blood stream and was excreted in the fæces.

NATURE OF BACTERIOPHAGE

Regarding the nature of the transmissible lytic agent there still is much controversy. Various theories have been put forward, but these essentially centre around two, namely (a) that the agent is a living ultra-microscopic virus (*q v*), and (b) that it is a non-living ferment supplied by the bacteria themselves. A third view, that bacteriophage is a gene, so far lacks experimental evidence. It is generally agreed that fixation of the phage by the bacteria is a necessary preliminary and that the actual lytic process depends upon an enzyme. The question at issue is whether this is produced by the bacteria themselves or by a definite exogenous virus. One has to note that such a virus has never been cultivated in any medium apart from bacteria in the living state. D'Herelle regards the change in the lytic properties which may be brought about in a bacteriophage as a process of adaptation and as evidence that the phage is a living organism. The phenomenon seems, however, explicable also on the enzyme theory, since if one enzyme can lead to the activation of another, a different or more active enzyme may be set free. Thus, those who regard the agent as non living consider that it is an endogenous autolytic enzyme transmissible from bacterium to bacterium. According to Bordet and Ciuca, the bacteria become modified in some way,

so that an inherent tendency to autolytic processes is exaggerated. This modification is transmitted to the descendants of the bacteria, and thus the lytic process is described as being hereditarily transmissible. The autolysis diffuses in the medium and sets up similar autolysis in other bacteria. Such a view involves the possibility of an enzyme giving rise to, or setting free, more enzyme of similar nature, as the lytic agent undoubtedly increases in amount. Bordet has pointed out as an analogy that thrombin in bringing about coagulation of the blood also leads to increase of itself. It has also been shown that trypsin and pepsin undergo a similar form of 'autocatalytic' increase (Kunitz and Northrop; Herriott and Northrop).

The particulate nature of phage has been demonstrated by physical methods. Thus, Schlesinger has caused the particles to sediment by powerful centrifuging and has estimated their size from the energy required to bring them down. Elford and Andrewes by the use of filters composed of collodion membranes of graded porosity, have shown that different phages which act on the same organism may vary greatly in size from 8 to 12 $m\mu$ to 50 to 75 $m\mu$. Investigation by means of the inactivating effect of X-rays on phages has given results as regards size which agree well with those of the ultra-filter and ultra-centrifuge (Wollman and Lacassagne). On the other hand, a particular phage does not undergo alteration in the size of its particles as a result of growth in different species of susceptible organisms or after purification.

Northrop has obtained a highly purified staphylococcal bacteriophage with the properties of a homogeneous nucleoprotein of molecular weight 300 million (as estimated by the ultra-centrifuge); it is very unstable.

A concentrated purified preparation of *B. coli* bacteriophage investigated by Schlesinger manifested practically no respiratory metabolism in relatively large amounts either by itself or with dead organisms belonging to the susceptible culture, or in a sub-lytic dose along with a living culture. No ferment property except phosphatase could be demonstrated, but other phosphatases derived from tissues were devoid of phage action.

Bacteriophage possesses antigenic properties which are distinct from those of the organisms on which it acts, thus phage-containing material when injected into animals develops antibodies which inhibit the lytic action. Schlesinger, by means of a purified *B. coli* phage, prepared an antiserum in rabbits which antagonised the lytic properties of the phage, but had no agglutinating effect on the corresponding organism, this, however, is exceptional. Bordet and Ciuca found that the antilytic effect is produced by antiserum which has been heated at 60° C; in this respect its action differs from that of bactericidal sera. The antiserum also agglutinates the phage particles. By means of such antisera,

between different phages which act on the antilytic effect is exerted only against the homologous phage. A phage which has been adapted to various species of organisms does not change its antigenic characters, i.e. its action on the original strain is still antagonised by an antiserum developed by it when present in any other strain.

The two questions posed at the beginning of this section have remained undecided—whether the bacteriophage is living or dead and whether or not it is purely a product of the organisms in the cultures developing it. The first has become merged in the wider problem of the nature of viruses. As has been seen, some of the latter, particularly those affecting plants, are so small, being of the size of a large protein molecule, that in regard to these the term life in the usual sense, as applied to organised structures, has no clear mean-

ing. As regards the second, the antigenic identity of a particular bacteriophage irrespective of the species of organisms in which it is propagated, has been advanced as evidence that phage is a distinct specific entity. However, the random manner in which a given antigen, *e.g.* Forssman's (*vide* p. 97), may be distributed among different animal species renders this argument inconclusive. Accordingly, taking into consideration all the facts, it does not seem justifiable at present to express a definite view as to the nature of bacteriophage

CHAPTER XXXII

THE PATHOGENIC FUNGI

By R. CRANSTON LOW

THE true fungi vary in size from the smaller forms—the unicellular yeasts—up through the multicellular moulds to the larger forms, such as mushrooms and toadstools. The latter come into the province of the botanist and need not be discussed here, the former are the concern of the bacteriologist and make up a very large group of organisms of various sizes and forms. Some are pathogenic to man and animals, whilst others, though non-pathogenic, are important in bacteriological technique because, being universally present in dust and air, they cause contamination of cultures and culture media. More recently also some of them have come into prominence on account of their power to produce antibiotic substances, such as penicillin.

DESCRIPTIVE TERMS APPLIED TO THE FUNGI (see Fig. 163)

The multicellular moulds are composed of elongated cells, arranged end to end in filaments known as hyphae (No 1). The whole mass is called the mycelium, which shows a loose network in the air. It is the vegetative part of the fungus. The main hypha of a mould is also known as a 'thallus' (θάλλος = γένεσις, τροφή). When mycelium grows the hypha is at first one continuous cell containing many nuclei at intervals in its course. It may remain like this or cross partitions (septa) may appear at regular intervals along its course, so that moulds are divided into those which have a non-septate and those which have a septate mycelium.

Although the hyphae grow by sprouting from the ends or sides of the cells, if conditions are suitable, fungi normally reproduce themselves by specialised cells called spores, which are reproductive organs and are not to be confused with the spores of bacteria. The chlamydospore, which is a resting stage, is the only fungus spore which has analogies to the bacterial spore.

The spores of fungi do not contain a true germ or plant embryo. They consist of an outer wall (epispore) and an inner wall (endospore), enclosing a mass of protoplasm containing droplets of fatty material and a minute nuclear body. There are a great many modes of spore formation, which are used as a basis of classification and identification. Spores may be either sexual, formed from two similar or dissimilar cells, by repeated division after the fusion of nuclear bodies, or asexual (conidia), formed by the division of a single cell without fusion with another. Some fungi reproduce themselves by only one process, others by both. In some parasitic fungi, such as the rusts, two different hosts may be necessary for the life cycle.

The different types of spores and spore-bearing organs are as follows:

Chlamydospores (No 4) are found in hyphae as large, rounded bodies with granular contents and a tough, thick, double-contoured wall. They may be terminal or interposed along the hyphae. They are formed by the division of a single cell without fusion with another. They are used to distinguish them from the sexual spores, formed either directly by division or budding, or on special

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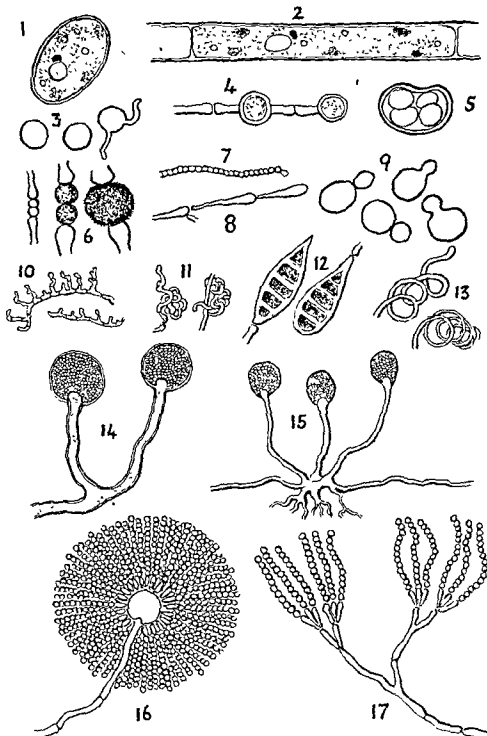


FIG. 163 (1) Yeast showing nuclear body, glycogen vacuole, fat globules, granular proto-
 (2) *Unguis* mycelium
 (3) Conidia (micro-
 m tubes 1 to 8 μ .
 staining 4 ascospores.

of sterigmata supporting rows of conidia (Nos 1-3, $\times 1200$), nos 4-9 and 12-17, $\times 450$; nos 10 and 11, $\times 150$)

hyphae (usually aerial) springing from the vegetative mycelium and bearing a single spore or group of spores. These special spores bearing fruit-bodies are:

Ascospores (No 5)

Oöspores are those

female spore (oogonium)

Zygospores (No 6) are the result of the union of two similar spores

Blastospores are formed by budding, e.g. in the yeasts. The bud is an exact replica in miniature of the original cell (No 9).

Arthrospores (No 7) are formed by the hyphae breaking up into short segments, which become disarticulated and so set free

Oidia are arthrospores of cylindrical form

Special spores (Macroconidia) are found in cultures of the Ringworm fungi and are characteristic of them. They are (a) *Fuseaux*, fusiform septate spores (No 12), with pointed or club-shaped ends (b) *Spirals*, with long or short coils (No 13) (c) *Pectinate bodies*, elongated elements with buds on one side only, producing a comb-like structure (No 10) (d) *Nodular organs*, consisting of twisted and knotted budding projections (No 11)

The significance and function of these special spores is quite unknown.

Vesicle is the swollen end of a hypha bearing a group of spores (No 16)

Sterigma (Nos 16 and 17) is a short stalk, bearing chain-like rows of conidia, e.g. in *Aspergillus* and *Penicillium*

Sporangium (Nos 14 and 15) is a sac containing an indefinite number of spores at the end of a hypha.

Sporangiophore is a special hypha bearing a sporangium (Nos 14 and 15)

Columella is the distal end of a hypha to which a sporangium (Nos 14 and 15) is attached

Stolon (No 15) is a runner-like branch of certain fungi, which runs along the surface of the medium

Rhizoids are root-like groups of projections occurring along the stolons and buried in the medium (No 15)

Sclerotia are resting bodies formed of compacted masses of mycelium and may be small or very large

GENERAL CLASSIFICATION AND BIOLOGY OF THE TRUE FUNGI (EUMYCETES)

Fungi are classified and identified by their methods of spore formation, so that it is not possible to identify a fungus in culture until spores are formed. Another method which is occasionally of assistance in identification is based on the fact that if two different fungi are grown together, so that the hyphae intermix, the mycelium of each, although intertwined, will never join up with each other. Whereas, if two cultures of the same fungus are allowed to grow together, the hyphal elements of each, in addition to intertwining, will meet end to end at their growing tips and fuse to form one continuous hypha. That is proof positive that these two fungi are identical.

EUMYCETES are divided into two main groups and five classes

(1) *Hyphae typically non-septate* = *PHYCOMYCETES*

(a) Sexual fructification by oöspores = *Oömycetes*
(b) Sexual fructification by zygospores = *Zygomycetes*

(2) *Hyphae typically septate* = *MYCOMYCETES*

(a) Principal spores endogenous in asci = *Ascomycetes*
(b) Principal spores exogenous in basidia = *Basidiomycetes*, e.g. *Mushrooms*
(c) Propagated by asexual spores (conidia) only = *Hyphomycetes* or *Fungi Imperfecti*

The majority of the fungi which are important to the medical bacteriologist are included in the *Ascomycetes* and the *Hyphomycetes*. The *Ascomycetes* produce fruit-bodies in a closed sac or ascus, which usually contains eight spores. They are the largest class of fungi, over 15,000 species having been described. In this class also are many common moulds and yeasts, both pathogenic and non-pathogenic, e.g. some of the *aspergilli* and *penicillia*, the yeasts used in brewing and baking and *Claviceps purpurea*, which produces ergot of rye.

The *Hyphomycetes* (*Fungi imperfecti*) are also a very large class with incomplete or incompletely known life cycles. The name *Fungus imperfectus* is not a good one, since it is not the fungus which is imperfect, but our knowledge of it which is deficient. So far as is known, they reproduce themselves by asexual spores (conidia) only, but many of them possess the characteristic mycelium of *Ascomycetes* and reproduce themselves by conidia similar to those formed by known *Ascomycetes*. Some of the *Aspergillus* and *Penicillium* groups produce ascospores and are therefore classified as *Ascomycetes*, whilst others, so far as is known, do not and these have been placed among the *Fungi imperfecti*. Most of the *Fungi imperfecti* are probably *Ascomycetes* which would produce the perfect stage with asci under special circumstances not as yet discovered. Many of the common fungi, popularly known as moulds and mildew, belong to this class, which also contains the fungi producing many of the common fungus diseases in man and animals, e.g. ringworm, thrush, etc.

Unicellular Fungi (Nos. 1 and 9) are popularly known as *Yeasts*, these are single-celled organisms which reproduce by budding only. They never produce true mycelium,

includes more coarsely granular masses staining with fuchsin and consisting of metachromatic material or volutin similar to the granules of the diphtheria bacillus. These volutin granules, consisting of material of the nature of nucleoprotein, must not be confused with the yeast-cell nucleus which, as in all fungi, is very small and does not stain with the ordinary basic nuclear stains. The nucleus can only be demonstrated after prolonged staining with iron hæmatoxylin, which stains it a black colour. This so-called nucleus is about 1μ in diameter. It has no definite chromatin network. Owing to its small size it is difficult to say whether it divides by karyokinesis, as some workers have stated. As it is so small and does not stain like an ordinary tissue-cell nucleus it is better not to call it a nucleus, but a nuclear body. When a yeast cell buds, part of the nuclear body is extruded from the parent cell into the bud to form the nuclear body of the new cell. Every yeast cell also shows a large, clear, highly refractile space or vacuole, which is filled with carbohydrate in the form of glycogen. When the yeast is growing under good nutritional conditions, the glycogen vacuole becomes very large, filling up a large part of the cell, but when the food supply is deficient, the glycogen is used up and the vacuole diminishes in size. This glycogen vacuole is very conspicuous in all yeasts when examined under the microscope in the unstained condition and is apt to be mistaken for the cell nucleus. In the cell cytoplasm there are also some small, very highly refractile fat globules.

Yeasts are divided into *True* and *False Yeasts*.

The *True Yeasts* or *Saccharomycetes* are used in baking, brewing, and distilling, and are never pathogenic. As they form ascospores they belong to the *Ascomycetes*. In culture they show a thick, opaque mucoid growth, smooth or wrinkled. They are white, cream or pinkish in colour and they grow rapidly at 37°C producing a very good growth in twenty-four hours.

The *False Yeasts* are the *Torula* or *Cryptococci*. They are of exactly the same shape and size as the true yeasts and, like them, reproduce by budding only. Their cultures are mucoid, but some of them are slimy in character. They are white, cream, yellow, or bright red in colour. As they produce no ascospores, they are classed among the *Hyphomycetes* (*Fungi imperfecti*). Most of the false yeasts are non-pathogenic and occur frequently in dust in the air, and on the skin, especially of the scalp. There is one pathogenic variety, the *Cryptococcus hominis* (*Torula histolytica*).

The *Multicellular Fungi* are those commonly designated 'moulds'. They occur in infinite variety and are divided into non-pathogens and pathogens. The non-pathogens include all the common moulds found on damp walls, stale bread, cheese, jam, etc. Their spores are universally present in dust and in the air and they do an immense amount of damage by causing spoilage of food, especially the moist foods such as fruits; they also contaminate culture media. Structurally, these moulds are similar to the yeasts, only instead of being round or oval, they are elongated in the form of the hypha. The hypha has an outer wall of fungus cellulose, granular protoplasm with minute nuclear body, volutin granules, glycogen vacuole, and fat droplets (No. 2). The fungus asexual spore (conidium) also has a similar structure to the yeast cell, only on a smaller scale. Hyphae vary in breadth from 1 to 10μ and the segments vary in length from 10 to 40μ , or even longer depending on the species. The spores vary in diameter from 3 to 10μ .

Intermediate between the unicellular yeasts and the multicellular moulds are those fungi which grow both by budding and by hypha production; there are two groups,

which differ in their cultural characters. In one group the culture is mucoid, e.g. *Monilia*, and in the other it is downy like the multicellular moulds, e.g. *Sporothrix*, *Hemyspora*, etc.

PHYSIOLOGY OF FUNGUS GROWTH

Nutrition The mould fungi, like the higher plants, produce vegetative growth (mycelium) when food is abundant and conditions favourable, and fruit bodies (spores) under conditions which tend to check growth. For fungus growth water and free oxygen are absolutely essential, also certain other chemical substances, e.g. sources of carbon, nitrogen, potassium, sulphur, phosphorus, magnesium, and sometimes iron. Very small quantities of food and moisture are required to support life. Fungi can grow on glass and metal surfaces, cotton and linen cloth, etc. They may get sufficient moisture, nitrogen, carbon, etc., from the air to allow growth and keep them in life. They obtain their nourishment by absorption of food materials in solution through the cell wall of the mycelium, which is submerged in the medium.

the air

Light. Most common moulds grow equally well in light or darkness. The growth of *Rhizopus nigricans*, a common contaminant, is stimulated by light. Other fungi are definitely phototropic, i.e. grow towards the light.

Temperature There is a wide range of thermal death-points, but on the whole, both spores and mycelium are less affected by dry than moist heat. Moulds are less resistant to heat than the majority of bacteria. As the fungus spore, unlike the bacterial spore, has practically no greater resistance to heat than the mycelium, all fungi are killed at 70° C. in a few minutes. The optimum temperature for growth varies with the species. Many common moulds, e.g. mucors and penicillia, grow best at 37° C., but many others, e.g. *sporothrix*, are inhibited at that temperature and grow best at room temperature, or slightly above it. Many species can live and grow below freezing-point. *Cladosporium herbarum* grows and its spores germinate on meat in cold store at -6° C. Ringworm fungi will also survive for months at that temperature. *Monilia albicans* will survive freezing at -79° C. for six months and probably much longer.

Reaction of medium A wide variation in the hydrogen-ion concentration is tolerated by fungi. In general, a slightly acid medium (pH 4 to 5) is favourable to rapid growth by fungi. In general, a slightly acid medium (pH 4 to 5) is favourable to rapid growth by fungi.

utilisation of nitrogen from sodium nitrate liberates alkali and so may... Moulds may change in colour when the pH of the medium changes. Fungi affect each other's growth when there is competition for available food. One group may break down organic material which can be utilised by another group, e.g. manure is broken down primarily by *Mucorales* followed by *Ascomycetes* and finally *Basidiomycetes* (toadstools). True parasitism of fungi on each other is not uncommon.

LABORATORY TECHNIQUE FOR DEALING WITH FUNGI This is much the same as for bacteria, but with certain modifications. Culture media are prepared and sterilised in the same way as for the ordinary bacteriological work and the same kinds of glassware are used. Fungi grow best in a medium containing sugar.

Czapek's medium is the liquid medium most frequently used by mycologists; it consists of

Saccharose	30.0	grms
Sodium nitrate	2.0	"
Dipotassium hydrogen phosphate	1.0	gm
Magnesium sulphate	0.5	"
Potassium chloride	0.5	"
Ferrous sulphate	0.01	"
Water	1,000	c.c.

The liquid is contained in flasks or flat screw-cap bottles lying on their sides and is inoculated with an emulsion of fungus mycelium or spores by means of a sterile pipette.

Solid media are more often used than liquids. For most purposes Sabouraud's medium is employed. It contains 4 per cent crude maltose or glucose and 1 per cent

or screw-cap bottles. This medium addition of gentian violet (1 in 500,000) or brilliant green (1 in 500,000) inhibit contaminating bacteria. Blood tellurite medium, as used for the growth of the diphtheria bacillus, can also be used, and MacConkey's medium is very useful for the

... wire-loop, but it must ... with the ... but it is ... medium ...

at 37° C., but more usually at ... As most fungi grow slowly the cultures must be kept for weeks before ... are obtained and therefore the lower the temperature the less drying of the medium. Screw-cap bottles are useful, but the cap must be removed at intervals to allow of aeration.

Some fungi keep their characters on prolonged subculture, but others lose their distinctive features. Mutation or sectoring (also called pleomorphism by Sabouraud) is ... Certain sectors of the culture change their appearance, ... the degree of sporulation. This change

breaks readily and the spores

The commonest ... her the ... al cases ... esses in ... ve also ... igus in ... condi- ...

Rhizopus nigricans (No 15) is continually met with in laboratory cultures. It is non-pathogenic. It has been described in cases of invader.

Aspergillus is also a common ubiquitous saprophyte, which contaminates cultures and wounds. The cultures are velvety and, when greenish in colour, somewhat like those of the penicillia, but they may also be whitish, yellow, or black. The hyphae are septate and asci are formed. The characteristic sporulation is by an unbranched conidiophore, which projects from the mycelium and has a swelling (vesicle) at its end, from which numerous sterigmata, each bearing a row of round conidia, are produced (No 16). These rows of conidia project in all directions, so as to form a large rounded 'head' which is easy to recognise.

and
path
from

A general infection of the internal organs may occur. The birds most often infected are the pigeon, canary, parrot, and penguin. Human beings are most frequently infected from the pigeon during the artificial feeding of infected squabs. The lung lesion of human beings resembles tuberculosis, and hæmoptysis and cavity formation may occur, but it usually runs a quicker course than tuberculosis. *Aspergilli* are frequently present in the sputum in asthma and chronic bronchitis, but, as in other lung infections, the presence of an *aspergillus* in the sputum does not necessarily mean that it is pathogenic. *Aspergilli* have also been found in infections of the external auditory meatus, nasal sinuses, vagina, eye, and skin, usually as secondary invaders. There is also a form of *Madura foot* associated with black granules in the pus, which is stated by Brumpt to be due to this form of fungus, designated *Aspergillus bouffardi* (*Madurella*). Primary infections of the brain with *aspergillus* have also been recorded, but these are very difficult to diagnose during life. In some cases, however, the fixation tests have given inconclusive results. Substances which are antibiotic to bacteria.

Penicillium is a very large genus of fungi, of which over 600 species have been described. Only some species are known to produce asci and these, therefore, belong to the *Ascomycetes*. *Penicillium* is one of the most easily recognised genera. The cultures are velvety and, although white at first, in a few days turn the characteristic bluish-green colour. When a plate colony of the fungus is examined under the low power of the microscope, the characteristic penicillium (No 17) can be readily seen. The penicillium or 'brush' consists of several sterigmata, each bearing a row of round conidia. This is a very common cause of spoilage in foodstuffs.

The great majority of fungus infections in man are infections of the skin and its appendages (Dermatomycoses), but there are also a number of other fungus infections of the viscera, which frequently lead to a fatal result. Within recent years, these latter conditions have been recorded with increasing frequency, indicating that they are probably not so rare as was at one time supposed.

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CLASSIFICATION OF FUNGUS INFECTIONS OF MAN

Group I : due to True Moulds, which show mycelium and spores in both tissues and cultures. Cultures show aerial hyphae and are never mucoid

Dermatomycoses	} (a) Microsporosis (b) Trichophytosis. (c) Epidermophytosis (d) Favus
(Tinea or Ring-	
worm)	

Aspergillosis (*vide* p. 618)

Coccidiomycosis

Chromoblastomycosis (' Mossy Foot ')

Group II : due to Yeasts and yeast-like fungi (Blastomycetes), which show only yeast-like forms with buds in both tissues and cultures. True mycelium is never found. Cultures are always mucoid

European Blastomycosis (Torulosis, Cryptococcosis)

Pityriasis capitis

Group III (Intermediate between Groups I and II) :

(a) *Due to fungi which show only yeast-like forms in the tissues and both yeast-like organisms and mycelium in culture. Cultures show aerial hyphae and are never mucoid*

Sporotrichosis

Hemisorosis

North and South American Blastomycoses.

Histoplasmosis

(b) *Due to fungi which show yeast-like organisms and mycelium in the tissues and yeast-like organisms (only or chiefly) in culture. Cultures are always mucoid*

Moniliasis (Thrush)

Pityriasis versicolor

Piedra

DISEASES DUE TO TRUE MOULDS

DERMATOMYCOSES

By far the commonest fungus infections of the skin, hair, and nails are the different forms of ringworm and favus. These diseases are spread by infection from man to man, animal to animal, or animal to man. The fungi of these conditions can be readily cultivated from the skin of infected human beings and animals, but, so far as is known, never occur as contaminants of media, so that they probably are true parasites and do not exist for long apart from the living animal body. *Epidermophyton* infection, however, may be spread by contact with infected clothing, or, in the case of the feet, by infected bathroom floors or mats, from which the fungus has been grown, but in these cases the contamination of the fomites has probably been a fairly recent one.

The Dermatomycoses are all due to the Fungi imperfecti (*Hyphomycetes*). The diagnosis can be confirmed by microscopic examination of material from
sufficient for clinical
human or animal, is

COLLECTION AND LABORATORY EXAMINATION OF MATERIAL In ringworm and *favus* of the skin the material for examination should be taken from the spreading edge of the lesion. The affected area should be cleansed with methylated spirit and scales from the edges be

on near the surface of the scalp and if necessary one must dig down with the forceps,

can be p^{...} applied.

arrangement The specimen is then arranged in a

the hig

sary.

fungus more visible. Also, it should always be remembered that when looking for fungus in any unstained specimen, if there is doubt as to whether what is seen is fungus or not, a negative report should be given. If fungus is present it is always quite typical and definite.

When cultures are to be made, the material is collected and placed on a slide, the instruments used and microscope slides all having been previously sterilised by flaming. The skin scales and pieces of nail should be washed with absolute alcohol or methylated spirit for one minute before being planted on the medium. This is not necessary nor advisable when dealing with ringworm hairs, which are placed on the sterile slide and, with a sharp sterile knife, cut into small pieces. The material is then transferred with a platinum loop to the medium, the sterilised loop having been previously pressed down into the medium so that the material will stick to it when touched by the wet loop.

weeks or so should be made, otherwise mutation may take place and the culture lose its characteristics. When grown on ordinary agar the culture does not tend to show mutation so readily as when on sugar agar. The appearance of the cultures will be described under each type of infection.

The staining of fungus in hairs, nails, and skin is rather unsatisfactory and difficult, and is unnecessary for diagnostic purposes.

If material from skin, hair, or nails has to be sent to a laboratory for examination and report, it should be placed between two previously flamed microscope slides, which are wrapped in a piece of white paper and placed in a small box.

RINGWORM or TINEA is classified according to the causal fungus, which may belong to one of four groups:

- (1) *Microsporon* (small-spore ringworm fungus);
- (2) *Trichophyton* or *Megalosporon* (large-spore ringworm fungus);
- (3) *Epidermophyton*;
- (4) *Achorion*.

Microsporon. The small-spore ringworm fungus is responsible for the majority of cases of ringworm of the scalp hairs in children in this country. When an infected hair is examined in liq. *t. cass.* (Fig. 161) it is found to be riddled with a fine septate mycelium (1 to 2 μ broad). As the fungus grows it splits up the hair substance, penetrates through to the surface where it forms bunches of small round spores (2 to 3 μ). The bunches of spores are so numerous that they form round the hair a complete sheath, which must

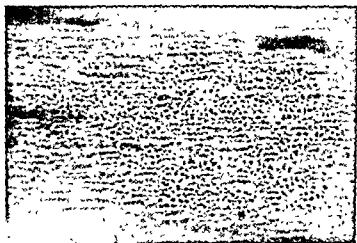


FIG. 164. Hair infected with *Microsporon audouinii*. Photograph of unstained preparation $\times 500$



FIG. 165. *Microsporon audouinii* on Sabouraud's maltose agar

be removed before one can see the mycelial threads underneath in the hair itself. As the mycelium pushes its way between the cells of the hair cortex, it separates them so that when a diseased hair is pulled on it never comes out by the root, but breaks off about the level of the skin surface. Where the hair breaks, the free end is characteristically frayed-out. In addition to infecting the hairs, the small-spore fungus may give rise to superficial red ringed lesions in the skin adjacent to the scalp. The nails are very seldom affected by this fungus.

The species of fungus most commonly present in the human small-spore ringworm is *Microsporon audouinii* (Fig. 165), but the cat fungus *M. felinum* is also a frequent cause. More rarely *M. lanosum* (dog) and *M. equinum* (horse) may be found.

All the small-spore ringworm fungi produce somewhat similar cultures, which show a flat, thin, radiating growth of a greyish colour with a fine

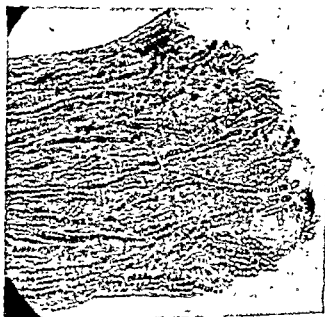


FIG. 166 Hair infected with *Trichophyton*. Photograph of unstained preparation $\times 500$

Note. The sizes of the 'spores' in Figs. 164 and 166 are not comparable, as in photographs of such thick preparations it is impossible to focus the outlines sharply.

velvety surface. They are never so thick and opaque as *Trichophyton*. On examining such cultures microscopically, small spores (microconidia) are seen springing from the sides of the hyphae, which in *M. audouinii* show racquet-shaped segments (Fig. 163, No. 8). The presence of thick-walled spindles (fuseaux) (Fig. 163, No. 12) with pointed ends is characteristic of *Microsporon*, and pectinate bodies may also be found.

Trichophyton or *Megalosporon* is associated with ringworm of the hair of scalp and beard and of the skin and nails. Infected hairs break off in the same way as in the small-spore infection and the nail substance is split up by the fungus growing in it. The skin lesions are more inflamed than in small-spore ringworm and show definite large rings with small blisters at the spreading edge. Deep granulomatous lesions which break down and suppurate also occur, especially in the animal infections, e.g. in ringworm contracted from cattle. On the scalp also, a large rounded area may swell up, becoming very red and tender to touch with pus oozing from every hair follicle. This is a deep infection with the fungus and is known as a kerion ringworm. Some-

what similar but smaller inflamed lesions also occur in ringworm of the beard. In all such cases the follicular suppuration leads to shedding of the diseased hairs and that, together with the allergy which develops, leads to a spontaneous cure of the condition in from eight to twelve weeks.

When hairs from trichophyton infections are examined microscopically, the fungus is found to have split up the hair substance as in the small-spore variety, so that the broken ends are brush-like (Fig 166). The fungus grows in long chains of rectangular slightly elongated elements called 'large spores'. These are probably short hyphal elements which have broken up to form rows

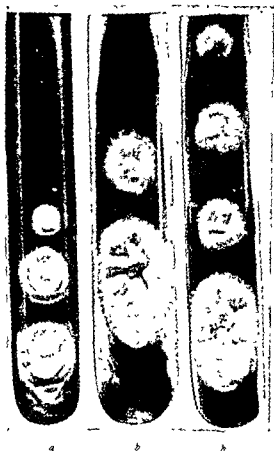


FIG. 166. *a*, *Trichophyton crateriforme*, *b*, *Trichophyton rosaceum*, Sabouraud's medium.

of arthrospores. They are slightly larger than the spores of *Microsporum*, being 5 to 8 μ in breadth. When the rows of spores run in chains in the substance of the hair, the fungus is known as *T. endothrix* and when outside on the surface of the hair, as *T. ectothrix*, and when in both situations, as *T. ecto-endothrix*. Large-spore ringworm fungus growing in the skin scales shows a network of long mycelial threads running in all directions, with no spores except an occasional chlamydospore. These threads of fungus do not follow the outline of the epithelial cells. When the nails are infected, the fungus is seen as rows of large square-cornered spores running in chains in all directions through the nail substance.

Cultures of *Trichophyton* are more luxuriant and opaque than those of the *Microsporum*. Many species of both human and animal origin, are known,

wrinkled and that of *A. quinckeanum* (Fig. 171, c) is white, raised, dome-shaped, and fluffy in appearance. Microscopically chlamydospores are found and peculiar macroconidia called 'chandeliers', which resemble pectinate bodies with short projections growing in all directions.

Immunity and Supersensitiveness in the Dermatomycoses. Favus does not occur so commonly as ringworm, because it is much less contagious. The hairs do not break off as in ringworm, in which the broken hairs and surface spores are shed on to the clothes, etc., of the patient. Also favus of the scalp does not die out spontaneously at puberty, as is the case in ringworm. Favus, if untreated, may last the whole lifetime of the patient, and lead to complete cicatricial baldness. Ringworm of the scalp, on the other hand, only affects children up to the age of about fifteen to sixteen years. At that age the disease dies out spontaneously whether it is treated or not. Similarly all young animals, e.g. kittens, calves, etc., are very liable to ringworm of the hair and in them the condition dies out spontaneously at the



FIG. 150. Photograph of drawing of scraping from favus scutellorum, showing spores and mycelium. (Crested, 1875)

age of twelve to eighteen months, which corresponds to fifteen to sixteen years of age in the human being. Adults may become infected with ringworm of the skin, nails, and beard hairs, but never of the scalp hairs. Why this should be so has never been explained. In addition to the more or less localised lesions produced by the fungi of ringworm and favus, generalised skin eruptions may occur. These are of several types—papular, vesicular, pustular, etc.—and they are accompanied by a general reaction with pyrexia, polymorphonuclear leucocytosis, etc. They may occur in all types of ringworm and favus infection, but are most commonly met with in the deep-seated infections, e.g. Kerion of the scalp, tinea barbae, and tinea corporis, due to an infection with cattle ringworm. According to the type of fungus producing the eruption they are known as microsporides, trichophytides, and favides respectively. The fungus of the original infection has been demonstrated in and cultured from these skin eruptions. It has also been cultivated from the circulating blood, but only during the first two or three days after the appearance of the skin rash. The fungus is carried by the

blood stream to the vessels of the

The

fa
is
of
have been made and are known as microsporin, trichophytin, and favin. They are in every way analogous to tuberculin. Polyvalent preparations of a mixture of microsporin, trichophytin, and favin are on the market under the name of 'trichophytin'. Bloch produced scarlatiniform and

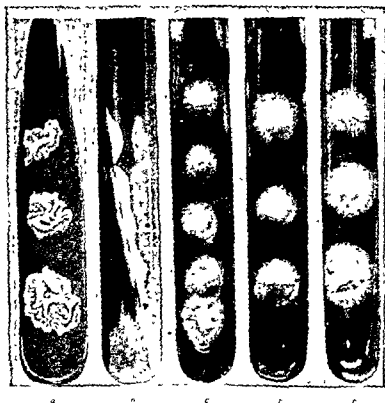


FIG. 171. a, Photograph of drawing of *Achorion schoenleinii* on Sabouraud's maltose agar. b, Side view to show elevation of growth. c, Photographs of cultures of *Achorion guineense*. (The central culture of c was isolated from a cat, and the two others from a man infected from it.)

lichenoid eruptions by intravenous injection of trichophytin in cases of kerion ringworm. When trichophytin is applied to the skin either by incision, by the Pirquet scratch method, or by intracutaneous injection, a positive reaction is given in all cases of deep-seated ringworm and favus infections. General reactive phenomena indicated by supersensitiveness and the presence of antibodies in the blood, are met with in fungus infections in general, but only when there is a considerable involvement of connective tissues, e.g. in deep-seated ringworm, and in infections such as sporotrichosis, hemisporosis, etc. (vide *infra*). The reactions are analogous to those which are found in other granulomatous infections, such as tuberculosis and syphilis. Evidence of supersensitiveness or allergy is obtained by injection of extracts of the parasite. For example, in deep-seated ringworm subcutaneous injection of trichophytin produces a local, a focal, and a

general reaction, the last being accompanied by pyrexia, etc. (cf tuberculosis). The presence of agglutinins, precipitins, and complement-fixing antibodies has been observed in several fungus infections. It is to be noted, however, that although all these reactions to fungus antigens are specific for fungus infections, they are not species-specific, so that they cannot be used as a means of identifying the fungus which is causing the infection. As in other antigen-antibody reactions, this absence of strict specificity indicates a certain degree of common antigenic structure in all the groups of pathogenic fungi.

In addition to the above reactions, which occur in microsporion, trichophyton, and favus infections, there is a special reaction which occurs in epidermophyton infections of the feet. In a considerable number of such cases, a sudden acute itchy vesicular eruption develops on the hands, especially on the palms and on the sides of the fingers. It is the same eruption as occurs on the hands and feet from other non-fungus antigens and which is known as cheiropompholyx. The epidermophyton fungus is never present in the hand lesions, but can always be demonstrated in the lesions of the feet. The eruption on the hands quickly disappears without any local treatment when the lesions on the feet are treated. This cheiropompholyx type of eruption is allergic in nature and probably a reaction between the fungus antigen absorbed from the foot lesion and the sensitised sweat glands of the hands. All deep-seated lesions of dermatomycoses lead to an allergy of the skin with positive skin reactions to the fungus antigen. A considerable number of quite superficial fungus skin lesions may also cause an allergy of the skin, but although attempts have been made to demonstrate agglutinins, precipitins, and complement-fixing antibodies in the serum of patients suffering from superficial fungus infection of the skin, they have never been successful. Only in cases with deep-seated lesions are these serum antibodies regularly found.

OTHER DISEASES DUE TO TRUE MOULDS (vide Fig. 172)

Coccidiomycosis (*Coccidioides granuloma*) is an infection due to a fungus called *Coccidioides immitis*. It occurs in two forms. (1) as an acute respiratory infection, which is recovered from and (2) as a chronic infection involving the skin, subcutaneous tissues, bones, and internal organs. The second form may lead to a fatal issue. The disease is chiefly found in North and South America, but cases have also been reported in Italy. Infection is believed to occur by inhalation of the fungus in the dust from the soil or through an injury to soil-contaminated skin. The disease occurs naturally in sheep, cattle, and dogs and also in rodents, so that these animals may be the source of the contamination of the soil. This view is supported by the fact that strains of *C. immitis* have been cultivated from the soil in areas where the disease is found.

The primary respiratory infection produces a mild type of lung involvement with very few symptoms or clinical signs, except slight cough. Allergic manifestations occur in two to three weeks in the form of Erythema nodosum and Erythema multiforme, arthritis, etc. As soon as these allergic phenomena develop, the tissue reactions cause a destruction of the fungus and the infection soon dies out. Patients who have this respiratory type of the disease very seldom show progressive involvement of the internal organs. All such cases give positive skin reactions to an extract of the fungus (*coccidioidin*). In areas where the disease is endemic, a considerable percentage of the population give positive skin reactions to the coccidioidin test, suggesting that they have recovered from previous subclinical infections.

Non-pulmonary coccidiomycosis most often occurs through a skin injury, usually on the hand. From the primary lesion there is a lymphatic spread up the arm, as in Sporotrichosis. Infection may also take place through the pharynx into the glands in the neck. From these lesions a general miliary spread may develop with rapidly fatal results, but more usually a chronic progressive condition occurs, with lesions of the internal organs and the bones and joints. This form of the disease is fatal, usually within a few months, but the patient may survive for a year or two. In the fatal cases, coccidioid meningitis is a frequent post-mortem finding. In the early stages of this type of

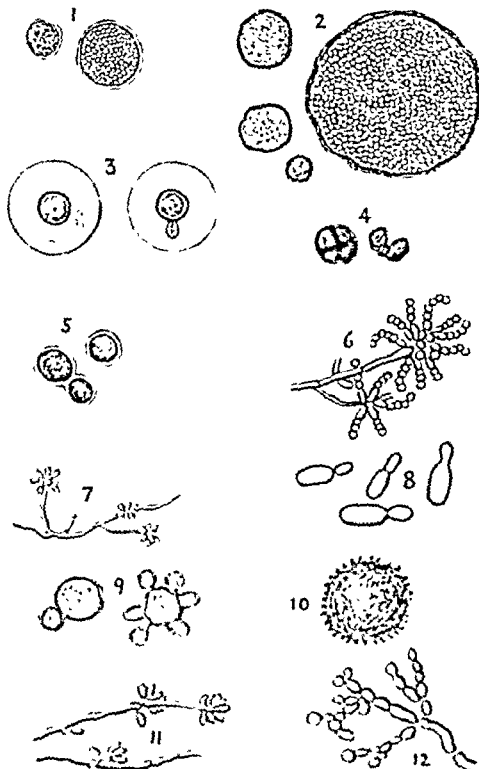


Fig. 1. A small, rounded cell with a granular interior. Fig. 2. A large, circular cell with a highly textured, granular surface. Fig. 3. Two large, circular cells, each containing a prominent, dark, circular nucleus. Fig. 4. Two small, rounded cells, one with a distinct nucleus and the other with a more granular interior. Fig. 5. Two small, rounded cells, one with a distinct nucleus and the other with a more granular interior. Fig. 6. A branching structure with multiple small, rounded cells at the tips of the branches. Fig. 7. A branching structure with multiple small, rounded cells at the tips of the branches. Fig. 8. Three elongated, rod-shaped cells, some with internal structures. Fig. 9. A large, rounded cell with a granular interior, and a smaller, rounded cell next to it. Fig. 10. A large, circular cell with a highly textured, granular surface. Fig. 11. A branching structure with multiple small, rounded cells at the tips of the branches. Fig. 12. A branching structure with multiple small, rounded cells at the tips of the branches.

chronic infection the intracutaneous test with a 1 in 1,000 'dilution' of standardised *t* in the terminal stages, as in advanced tuberculosis severe cases complement-fixation and precipitin tests sent These skin and serum antibody reactions are Where material can be obtained, it is

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an abundant aerial mycelium, which at first is grey-white, but later becomes brownish in colour The mycelium shows septate branching hyphae with rectangular or spherical arthrospores When injected into the testis of the guinea-pig, it produces a severe orchitis within a week or ten days Mice can also be infected by intraperitoneal injection of the culture mycelium

Chromoblastomycosis or Verrucous Dermatitis is caused by one of three fungi called *Hormodendrum pedrosoi*, *Hormodendrum compactum*, and *Phialophora verrucosa* The clinical lesion begins as a small warty elevation of the skin, from which it slowly spreads to produce warty papillomatous lesions, which sometimes show areas of ulceration if secondarily infected The warty lesions are dark reddish-blue in colour, thickened and infiltrated at their bases and they project above the level of the skin as greyish, warty excrescences The condition usually begins on the back of the foot and from its appearance is often known as 'Mossy foot' It may, however, start on the legs, hands, arms, chest, buttocks, or face It is found chiefly in North, Central, and South America, Russia, North and South Africa, Japan, Sumatra, and Java. It does not occur in Great Britain It very often starts after an abrasion of the skin of an exposed part with some kind of wood It does not spread from man to man When it begins it is usually unilateral and it spreads very slowly, years may pass before an entire limb is affected The general health is not interfered with and, unless secondarily infected, the lesion is painless The disease is usually confined to the skin and subcutaneous tissues, but has been recorded in muscle The causal fungus is found by direct examination of pus or crusts from the warty lesions It shows as dark brown bodies, hence the name Chromoblastomycosis These bodies are single or in bunches, rounded in shape, surrounded by a thick outer wall and divided into compartments by septa (No 4). They multiply by fission and never show budding. On biopsy these bodies are found in the corium of the skin in small abscesses or in giant cells. There is an inflammatory granuloma round them and often considerable cicatricial change in the dermis and subcutaneous tissues Through the chronic inflammatory process in the dermis the epithelial layers become hypertrophied and so the warty surface is produced The brown bodies in the tissues appear identical whichever of the three fungi already mentioned is the causal one and it is only by culture that one can tell which fungus is responsible for the condition When material from the lesions is placed on Sabouraud's medium growth occurs readily at room temperature All three produce slowly growing colonies, dark brown or black in colour, with a surface velvety from aerial spore-bearing hyphae On microscopic examination of the cultures *Phialophora verrucosa* shows a septate mycelium with flask-shaped conidiophores growing either terminally or laterally from the mycelium These conidiophores have a cup-shaped tip which is surmounted by bunches of oval conidia (1.5 to 4 μ in length) There is no other form of spore formation in this fungus The *Hormodendrum*, however, show three types of sporulation: (a) the *Hormodendrum* type (No 12), characterised by conidiophores bearing conidia in branching chain formation, (b) the *Acrotheca* type in which swollen, club-shaped conidiophores bear conidia along their sides (No 11), and (c) the *Phialophora* type with spore formation as in *P. verrucosa* These three types occur in varying proportion, according to the strain of the fungus and the medium used *Hormodendrum compactum* is so called because of the arrangement of the chains of the conidia in compact masses, the *Phialophora* type of conidiation is also seen in this fungus Laboratory animals do not develop warty lesions

negative The typical warty skin lesion has been produced in man by injection of the living culture Complement-fixing antibodies have been demonstrated in the sera of cases of Chromoblastomycosis The sera of the cases due to *H. pedrosoi* in the U.S.A. also fixed complement with strains of *H. pedrosoi* from cases in South America, and in cases due to *H. pedrosoi* there was cross-fixation with strains of *P. verrucosa*, indicating a common antigenic factor in both fungi.

The disease produced by this yeast is *Cryptococcosis*.

Although it is called European and South American Blastomycosis, it is South America. This infection is a

true blastomycosis, as the parasite occurs only as a budding yeast both in the tissues and on artificial culture. Mycelium is never produced. The culture on Sabouraud's medium is a semi-transparent, slimy, yellowish, or brownish growth of a sticky character. The lesions which it produces are subacute or chronic and may occur in the skin, subcutaneous tissues, and viscera, especially the lungs, but the fungus also has a marked preference for the brain and meninges, which are frequently affected. Infection probably takes place through the respiratory tract. The diagnosis is made by finding the yeast in the sputum or in the

glands. The yeast is ovoid or capsule, giving the appearance of a clear zone round it, hence the name *Torula histolytica* (No. 3). Many of these bodies show buds. The same bodies occur in the lungs, brain, etc., and in cultures. In the tissues there may or may not be a great cellular reaction. When present, it occurs as a chronic granuloma with giant cells, some of which may contain the parasite. In the lymphatic glands a cellular reaction resembling Hodgkin's disease may be found and in subcutaneous lesions myxomatous changes are sometimes seen. The fungus grows readily on Sabouraud's medium, producing a mucoid or slimy growth, which is white at first, but later becomes cream-coloured or brownish. Microscopically the yeast can be seen, with or without buds, to have a thick capsule, which can be easily demonstrated by the nigrosin method. As it grows it may show short germ tubes, but never true mycelium.

There are a few reports of agglutinating and complement-fixing antibodies in the sera of infected persons, but in many cases they cannot be demonstrated. Local and focal reactions to cryptococcus antigens have been recorded, showing that allergy has been produced. Skin tests have not been made in a sufficient number of cases to form an opinion as to their value in diagnosis. The diagnosis can usually be easily made by microscopic examination of the sputum, cerebro-spinal fluid, etc. This can be confirmed by culturing on Sabouraud's medium and by intraperitoneal inoculation of rats or mice.

For the purpose of staining, the yeast is washed with distilled water, then with 1% of sodium acetate, and then stained with Leshman's stain, in addition to cocci and diphtheroid bacilli, etc., numerous large usually clumped together into colonial 'bottle' bacillus. It is now generally

Many attempts have been made to cultivate this yeast. Success has been claimed on beer-wort agar and on media containing a high percentage of glycerol and it is reported to have grown as an oval yeast exactly as on the scalp. The cultures obtained were moist, smooth.

The writer has been unable to cultivate the yeast on any of the media tried, but it is always present in dandruff of the scalp.

DISEASES DUE TO FUNGI WHICH SHOW YEAST-LIKE FORMS IN THE TISSUES AND YEAST-LIKE FORMS AND MYCELIUM IN CULTURES

Sporotrichosis is a chronic infection with a fungus called *Sporothrix schenckii*. It was first described by Schenck in 1892.

It always occurs in persons living in the country and in constant contact with animals. Horses suffer from it and it has also been reported, but less frequently, in dogs, cats, rabbits, and rats. The infection occurs in two forms. (1) the localised infection of skin and subcutaneous tissues and lymphatic vessels of a limb, and (2) the disseminated type affecting the subcutaneous tissues, bones, etc.

The localised type, which is the common one, usually starts from a scratch on the hand, where a primary lesion (chancre) develops as an indolent bluish-red nodule, which

slowly enlarges, softens, and bursts to form an indolent ulcer. Soon secondary nodules of a similar character appear in the course of the lymph vessels, forming a chain of nodules at intervals spreading up the limb. The condition may last for months or years. The lymphatic glands of the area become involved, but only rarely is there blood-spread to internal organs. Many cases have occurred among miners in the Transvaal.

In the disseminated type there is usually no detectable primary lesion. As a rule, the first thing noticed is the sudden appearance of multiple hard subcutaneous nodules scattered over the body. They do not usually ulcerate unless incised. The condition begins acutely with pyrexia and the patient may die in a few weeks or months. Vegetative and ulcerative lesions may also occur in the mucous membrane of the nose and mouth. Bones, joints, muscles, and tendon sheaths may be affected, but visceral lesions are rare. Sporotrichosis differs from other mycotic infections in that the lung is rarely infected, but when a pulmonary infection does occur the sporothrix fungus can be found in the sputum.

Microscopic sections of sporotrichosis lesions show a granuloma with giant cells. Between the cells oval yeast-like bodies with buds may be seen. Where they occur in clumps they are relatively easy to recognise, but as they are often scattered, they look

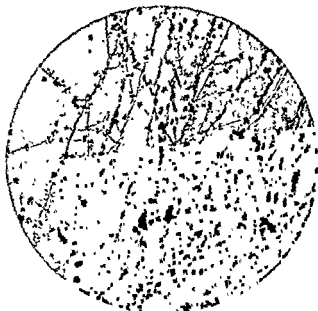


FIG. 174. Edge of living colony of *Sporothrix schenckii* on agar, five days at 22° C. $\times 200$

not unlike fragments of the nuclei of pus cells. The fungus can be grown from the skin ulcers, but in order to avoid bacterial contamination, if possible, an unbroken nodule should be punctured and cultures made on Sabouraud's medium. They should not be incubated because only a scanty growth of yeast-like forms occurs at 37° C. (Fig. 175), whereas at room temperature in a few days a white velvety colony appears. It slowly increases in size and later becomes wrinkled and dark brown in colour. Culture is the best method of making a diagnosis but pus or other material may also be injected intraperitoneally into a male white rat, when the Straus reaction with a marked orchitis is produced.

Microscopically the culture at room temperature shows a narrow, branching septate mycelium (2 μ in breadth) with lateral oval conidia and side branches of short hyphae tipped with a bunch of oval or pear-shaped spores (Figs. 172, No. 7 and 174). Patients with sporotrichosis frequently give a positive skin reaction to sporothrix antigen (sporotrichin). Positive agglutination and complement-fixation reactions also occur with the sera of patients with sporotrichosis, the antigen being prepared from spore suspensions of the fungus.

Hemysporosis is a fungus infection due to *Hemyspora stellata* first described by Gougerot and Lévain in 1909 in France. Since then a considerable number of cases have been reported, especially from Italy. The condition has many characters in common with sporotrichosis. Similar granulomatous nodules which occasionally break down

and ulcerate, occur in the skin and subcutaneous tissues. These may be single or multiple and are usually confined to the skin, but the bones are occasionally affected. The fungus is present in the granulomatous masses as yeast-like bodies, but on microscopic examination of sections, as in sporotrichosis, the fungus is not always easy to detect, so that cultures should be made on Sabouraud's medium. The culture, which is velvety, is at first whitish, but later becomes brown and wrinkled. Microscopic examination of the culture shows a septate mycelium and a star-like arrangement of the conidiophores growing from the mycelium, hence the name *H. stellata* (No 6). Some of the mycelial threads also become swollen at their free extremities where 'protoconidia' are found. These again segment into three or four almost spherical 'deuteroconidia'. *H. stellata* also produces oöspores and therefore belongs to the Oömycetes. The sera of patients with Hemisporosis give positive agglutination and complement-fixation reactions with suspensions of the parasite. These, however, are not strictly specific, as reactions may also be obtained with other fungi. It must be remembered that this fungus may occur as a contaminant, since it is fairly common in the dust of rooms, etc.

North American Blastomycosis (Gilchrist's Disease) is a chronic infection due to *Blastomyces dermatitidis*, on artificial culture grows



FIG. 175. Film from agar culture of *Sporothrix schenckii* grown at 37° C. for ten days. Gram's stain $\times 1,025$.

From the lung the fungus becomes disseminated to other parts of the body, such as bones, brain, spinal cord, skin, etc. Very often subcutaneous gummatous-like lesions or abscesses appear, which burst to the surface and ulcerate. The systemic form of the disease is practically always fatal.

but a few sporadic cases have been recorded from time to time in Canada, Europe, and elsewhere. Owing to incomplete investigation it is doubtful whether all the cases recorded under this name were true examples of the disease. There are two types of the infection—the cutaneous and the systemic disseminated types.

Cutaneous blastomycosis is the disease which follows inoculation of the fungus through the skin. It nearly always begins on the exposed parts, especially the face, hands, and feet. It begins as papular or papulo-pustular lesions, which break down and crust over, assuming a raised rough warty appearance with pus oozing from between the papillomatous masses. It gradually spreads to form large patches, which continue to extend at the edges whilst healing in the centre with marked scarring. The condition looks not unlike the warty form of *ekra* and it is cutaneous internal tissues and does not tend to spread to internal organs.

In *systemic blastomycosis* infection usually occurs through the respiratory tract, producing in the lung a condition suggesting tuberculosis disseminated to other parts of the body, such as bones, brain, spinal cord, skin, etc. Very often subcutaneous gummatous-like lesions or abscesses appear, which burst to the surface and ulcerate. The systemic form of the disease is practically always fatal.

capsules
tissues

as with *S.*

grows as a fluffy white colony with aërial hyphae. Later the colour changes to a brownish black. Microscopic examination of the culture shows the same yeast-like bodies as are found in the tissues, together with a septate mycelium with round conidia attached to the sides of the hyphae either directly or by lateral stigmata. Chlamydo-spores are produced. If the culture is incubated at 37° C. only yeast-like forms are produced. The disease is a chronic addition of tuberculous lesions with emulsions of

extracts of *Blastomyces dermatitidis*. This reaction is quite specific, being present only in blastomycosis and never occurring with other fungus antigens. No complement-fixation reaction occurs with sera, even when undiluted, of patients with only localised cutaneous lesions. Most cases of blastomycosis, in whichever form it is present, give skin reactions to the fungus or its products. The reaction is of the same type as is produced by tuberculin and never occurs unless the patient has blastomycosis. In the later stages of the disease it may be absent.

When suspensions of the cultures are injected intraperitoneally into rabbits, guinea-pigs, rats, and mice, typical lesions containing the fungus in budding, yeast-like forms, develop in the liver, spleen, lungs, etc. (Fig. 177).

South American Blastomycosis (*Paracoccidioides Granuloma*) only occurs in South America and is a chronic infection due to *Blastomyces brasiliensis*. It can be divided into four types according to the site of infection: (a) muco-cutaneous, (b) lymphadenitic, (c) visceral, and (d) mixed.

The muco-cutaneous form is found in the lips, gums, tongue, palate, inside of cheek and nose, and in the skin of the face, usually near the mouth. It commences as a small papule, which quickly breaks down and ulcerates. As it spreads peripherally it causes considerable destruction of tissue. The regional lymphatic glands soon become infected and from them, through the blood stream, other lymph glands, these swell and rupture externally, producing more skin lesions. The lymphadenitic type usually occurs apart

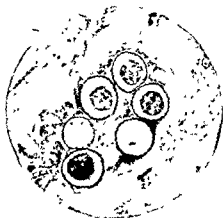


FIG. 176. Double contoured bodies in tissues from one of Rivford and Gilchrist's cases of North American Blastomycosis $\times 500$



FIG. 177. *Blastomyces dermatitidis*. Growth of yeast-like organism in kidney of rabbit infected from human case $\times 1,000$

from skin lesions and as the gland enlargement is usually in the neck, infection presumably occurs from the naso-pharynx. The lesions tend to burst through the skin and produce discharging sinuses. The visceral type is caused by infection through the intestinal canal. It occurs as deep ulcers in the lymphoid tissue of the gut, especially in the region of the caecum and appendix. From the bowel a blood-spread takes place to the liver, spleen, kidney, and lungs. The mixed type shows a combination of the above three types.

Material can be obtained easily from the muco-cutaneous lesions, lymphatic glands, or sputum. When examined direct in liq. potassae it shows *Blastomyces brasiliensis* as single and multiple budding yeast-like cells (10 to 60 μ) with thick walls. The single cells are identical with those seen in North American Blastomycosis, but cells with multiple buds in clusters are characteristic of South American Blastomycosis (No. 9). The buds are often quite small being only 1 to 2 μ in diameter. Cultures should be made on Sabouraud's medium or blood agar. When incubated at 37° C. wrinkled mucoid colonies result consisting of single and multiple-budding yeast-like bodies, the same as are found in the tissues. When kept at room temperature most strains develop aerial mycelium and produce a velvety white growth, which turns brown with age. Microscopically the culture fungus is very similar to that seen in North American Blastomycosis except for the presence of multiple budding yeast-like bodies. Microscopic examination of lesion-formations shows granulomatous formations with giant cells, necrosis and abscess formation. They are very similar to those described under North American Blastomycosis and to Coccidiomycosis, hence the disease has sometimes been called Paracoccidioid Granuloma. The sera of patients with South American Blastomycosis have

given positive complement-fixation reactions with fungus extracts, but Fonseca found that broth culture filtrates of *Blastomyces brasiliensis* also gave positive complement fixation reactions with sera of patients suffering from sporotrichosis, epidermophytosis and chromoblastomycosis. Intracutaneous injections of culture antigens produce focal reactions with redness and swelling of the lesions and in some cases a general reaction with pyrexia.

Material from lesions or culture suspensions when injected intraperitoneally into

is found in North, South, and Central America, South Africa, the Philippines, and Great Britain. Since the condition has been found as a spontaneous infection in the dog, it has been suggested that infection, especially in children, may occur from that source. The disease has some resemblances both in the clinical and microscopic findings, to Kala-azar. There is pyrexia, loss of weight, anaemia, leucopenia, and enlargement of the liver and spleen. The lymphatic glands are also usually enlarged and in generalised cases the lungs are practically always affected. Bones and joints may be involved and skin ulceration may occur. Oral and pharyngeal lesions have also been reported.

The diagnosis is made by examination of blood films, smears from splenic or sternal puncture, or from excised lymphatic glands in which the parasite is found. The condition is

tically Gram's

as numerous small, round, pinkish-brown bodies, which are slightly smaller and do not show the characteristic

Leishman-Donovan bodies, but are slightly smaller and do not show the characteristic

addition there are characteristics which are diagnostic of histoplasmosis. In the few cases examined, complement fixation tests have been made in this condition. In one case a positive intracutaneous reaction was obtained with a filtrate of a broth culture of *H. capsulatum* and Palmer obtained positive skin reactions with an extract of the fungus (histoplasma) in the milder forms of the disease. Injection of the fungus intraperitoneally into guinea-pigs and mice produces visceral granulomatous lesions from which it can be recovered.

DISEASES DUE TO FUNGI WHICH SHOW YEAST-LIKE ORGANISMS AND MYCELIUM IN THE ISSUES AND YEAST-LIKE ORGANISMS (ONLY OR CHIEFLY) IN CULTURE

Moniliasis (Thrush) is a subacute or chronic infection of the mucous membranes of mouth and vagina, the skin, nails, respiratory tract, and lungs, due to a fungus called *Monilia albicans* (*Candida albicans*). This fungus grows in the tissues in yeast forms with buds and abundant mycelium, but in cultures, which are mucoid and are never downy, almost entirely in yeast form. The disease occurs in all races and at all ages.

Oral thrush, which is the commonest form of the condition, occurs most often in infants and in elderly persons suffering from wasting diseases such as tuberculosis or

The infection may spread to the tongue, gums, and inside of cheeks. They

perleche

occurring in 10 to 15 per cent of women. The frequency of vaginal thrush in 50% of the vagina at that time, together with vaginal epithelium. In persons whose occupation entails consequent maceration of the skin, it

may be localised to the hands, and is especially common among fruit packers. It affects the skin of the backs of the hands and of the fingers, especially in the interdigital spaces. It may also infect the nails, which are thickened, of a greenish or brownish colour, and often raised off the nail bed. A chronic paronychia with redness and swelling at the base of the nail due to an infection with *M. albicans* between the nail and the skin fold may also occur. Similarly, moniliasis may occur on the foot between the toes, spreading from there to the adjacent areas. As the skin between the toes becomes sodden-looking, it may resemble an epidermophyton infection. An intertriginous infection with sodden oozing patches may also occur under the breasts in women or in the axilla, groins, or gluteal folds. A whitish, itchy area of macerated skin may also occur round the anus. Occasionally a more or less generalised moniliasis may affect large areas of the skin, and fatal cases of this type have been recorded.

Respiratory moniliasis. From the mouth moniliasis may spread down the oesophagus, especially in young infants, the fungus growing in long mycelial threads right through the mucous membrane into the muscular layer below. These cases often end fatally. When a bronchial infection occurs it produces in adults a chronic form of bronchitis with persistent cough and mucoid sputum which contains abundant fungus. This form of the disease may last for years without seriously affecting the general health. Pulmonary moniliasis is a rarer condition, but much more serious. The lesion is of broncho-pneumonic type and in young infants is very fatal.

Moniliasis of the bones and joints has also been recorded and **endocarditis** similar to subacute bacterial endocarditis occasionally occurs.

The diagnosis of moniliasis is made by demonstrating the fungus in material from the mouth, skin, nails, sputum, etc. The membranous material from the mucous membrane lesions of the mouth and vagina should be examined in liq. potassæ, when a felted mass of septate mycelium with numerous rounded or oval yeast-like bodies many of which show buds, will be found together with epithelial and pus cells. Large chlamydospores are also frequently seen. Similarly in oesophageal, pharyngeal, bronchial and pulmonary infections the fungus is found in abundance in the sputum. It usually occurs chiefly in oval yeast-forms with buds, but definite short hyphæ are often also present. In infections of the respiratory tract the fungus can usually be found in the faeces. It must always be remembered, however, that the presence of *M. albicans* in the sputum or faeces must not be taken as proof of monilial infection. It occurs in the sputum and faeces where there is no visible sign of moniliasis, and in lung conditions especially a diagnosis of moniliasis must only be made after repeated examinations and after the exclusion of all other possible conditions, such as tuberculosis.

Cultures should be made from swabs or scrapings of the mucous membrane lesions or from the sputum or faeces on Sabouraud's medium and incubated at 37°. Quite a good growth occurs within twenty-four hours. The colonies are creamy white, moist, glistening, and mucoid. With age they become slightly wrinkled and brownish in colour. Microscopically the cultures, especially if incubated, show nothing but rounded yeast bodies with buds, but large chlamydospores are also numerous. In older cultures some mycelium may develop. When it is important to identify the fungus definitely a rabbit should be inoculated intravenously with 1 c.c. of a 1 per cent suspension of the fungus. Death occurs in four to five days with whitish miliary lesions in the internal organs, especially the kidney. These lesions show abundant fungus in yeast form and short hyphæ. *Monilia albicans* is the only member of the monilia genus which is pathogenic for laboratory animals.

A great many species of monilia have been described and their classification at the present time is in rather a confused condition. A monilia like *M. albicans* has been found in the stools in sprue and at one time thought to be the cause of that condition. Similarly in intertriginous affections of the skin and in paronychia the monilia found has been described as *M. pinoyi*. Both these forms may be different strains of *M. albicans*. Attempts have also been made by Castellani and others to classify the monilias by their sugar reactions, but these are so variable that they are of little value. The exact botanical position of the monilias is not settled but the fact that they do not produce spores distinguishes them from the *Endomyces*. The following table gives



FIG 178 *Monilia albicans*. Film from culture stained with fuchsin. $\times 500$

the distinguishing features of the yeast-like fungi when grown on Sabouraud's medium.

Saccharomyces (True Yeast)	Torula or Cryptococcus (False Yeast)	Endomyces	Monilia
No mycelium	No mycelium	Mycelium	Mycelium
Ascospores	No ascospores	Ascospores	No ascospores

Microscopically, sections in cases of monilia infections of mucous membrane, skin, internal organs, etc. all show a granulomatous structure and necrosis of tissue. The fungus is present both in yeast form with buds and as branching mycelium.

Immunity and allergic reactions Agglutinins have been demonstrated in high titres in the sera of patients with moniliasis, but the reaction may be absent even in severe infection.



FIG. 179 *Malassezia furfur*, scraping from skin. Gram's stain $\times 200$.

from one of the brownish areas and examined in liq. potassae, there is no difficulty in seeing the fungus (Fig. 179). It occurs as a septate mycelium (3 to 4 μ broad) lying on and between the epithelial cells. The mycelium tends to break at the septa into short curved segments (7 to 12 μ long). There are also clumps of round yeast-like spores many of which have spherical buds. The clumps of spores are often arranged characteristically like bunches of grapes. The fungus can also be demonstrated with Leishman's or Gram's stain, but for diagnostic purposes examination in liq. potassae is sufficient.

Malassezia furfur has never been cultivated satisfactorily. A few workers claim to have grown this organism in culture, but all found it impossible to secure continued growth. The cultures obtained were mucoid in character, brownish in colour with slight 'hairiness' at the periphery. After many attempts on all the media recommended and many others, the writer has completely failed to obtain a growth of any kind.

Piedra (Trichomycosis Nodularis) is a fungus infection of the shaft of the hairs of the scalp, beard, and moustache. It produces stone-hard nodules firmly attached to the hairs. There are two varieties of the condition—Black Piedra, confined almost exclusively to certain tropical regions, and White Piedra, which is rarer and may occur in both the tropics and temperate regions. In the latter the nodules are of a yellow-brown

with the different species of monilias are not species-specific. In cases of moniliasis, positive skin reactions occur to fungus extracts (oidiomycin) on intracutaneous injection, but a considerable number of apparently normal individuals give positive reactions to this fungus extract. This may be due to the fact that, as already mentioned, *M. albicans* can be isolated from the sputum, faeces, or skin of many normal persons. In general

hands.

Pityriasis Versicolor is a skin condition in which a saprophyte *Malassezia (Microsporum) furfur* grows in the horny layer of the skin of the body and limbs producing areas of a light brown colour and slight scaliness. The condition is widespread all over the world; in this country it is not so common as it used to be. If scales are scraped off with a knife

smooth and mucoid and contains septate hyphæ, chlamydospores, and asci similar to those found on the hairs

White Piedra is due to a different fungus, namely *Trichosporon beigeli*. The nodules consist of a felted mass of mycelial threads and blastospores, but no asci. On culture the fungus shows a soft, slimy, cream-coloured growth, which later becomes wrinkled.

Piedra should not be confused with the condition found commonly in Europe on the hairs of the axillæ and pubic areas and called *Trichomycosis nodosa*. The nodules in this condition are not so hard as in *Piedra* and they are due to a streptothrix, *Nocardia* (*Actinomyces*) *tenuis*.

(Black or Hairy Tongue) is a rare condition whose etiology is still doubtful, it has been stated to be due to a fungus. The centre of the posterior part of the tongue is covered with brownish-black long papilliform projections. Several organisms have been found in these cases. They include three types of cryptococci, a streptothrix, a leptothrix, *Rhizopus nigricans*, and chromogenic cocci. There is not sufficient evidence to show whether any of these findings is significant. Recent investigations suggest that the condition may be due to a vitamin B deficiency.)

CHAPTER XXXIII

PATHOGENIC PROTOZOA—MALARIA PLASMODIA; BABESIA (PIROPLASMA); THEILERIA

MALARIA (French, paludisme) is the most prevalent infection of man, being specially frequent in tropical and subtropical countries. It was identified in antiquity by the regularly recurring febrile attacks which characterise certain forms of the disease, and later the specific effect of quinine in abolishing the fever afforded a therapeutic method of diagnosis. The action of malaria in producing a great variety of disease manifestations and in conducing to racial degeneration has also been recognised. It has now been completely established that the malarial fevers are protozoal infections, there being different forms of the parasite. These belong to the *Hæmosporidia* (an order of the *Sporozoa*), which are blood parasites, infecting the red corpuscles of mammals, reptiles, and birds. The parasite of human malaria is generally known by the generic name *Plasmodium*, and three well-defined species have been recognised: *Plasmodium vivax* of Benign Tertian Malaria, *Plasmodium malariae* of Quartan Malaria, and *Plasmodium falciparum* (or *Laverania malariae*) of Malignant Tertian Malaria; *Plasmodium ovale* is probably a fourth species. The malaria parasite was first observed by Laveran in 1880, and his discovery has received confirmation from the researches of many workers in various parts of the world. Golgi supplied important additional information, especially in relation to the sporulation of the organism and the varieties in different types of malarial fever. MacCallum (1897) observed *in vitro* the fusion of the flagellate form of the parasite with the rounded form and recognised the sexual nature of the process. To Manson especially belongs the credit of regarding the exflagellation of the organism as a preparation for an extra-corporeal phase of existence. By induction he had arrived in 1894 at the conclusion that the cycle of existence outside the human body probably took place in a mosquito. It was specially in order to discover, if possible, the parasite in this insect, that Ross commenced his long series of observations, which after patient and persistent search were ultimately crowned with success. He found rounded pigmented bodies in the wall of the stomach of a dapple-winged mosquito (a species of *Anopheles*) which had been fed on the blood of a malarial patient. The pigment in these bodies was exactly similar to that in the malaria parasite, and he rejected the possibility of their representing anything else than a stage in the life cycle of the organism. He confirmed this discovery and obtained corresponding results in the case of the *Proteosoma* (*P. præcox*) infection of birds, where the parasite is closely related to that of malaria. He was able to trace all stages of the development of this organism from the time it entered the stomach of the insect along with the blood from infected birds, till the time when it settled in a special form in the salivary glands. Ross's results were published in 1897 and 1900. His discovery has not only elucidated the mode of infection, but, as combating the

in the case of the different species of the human parasite by Manson and Bastianelli, and these with other Italian observers also provided important information regarding the transmission of the disease by infected

mosquitoes. Koch's observations emphasised that human malaria depends solely on the circulation of the parasite between mosquito and man. Lastly, mention should be made of the striking experiment carried out by Manson by means of mosquitoes fed on the blood of patients in Italy suffering from mild tertian fever. The insects after being thus fed were taken to London, and allowed to bite human subjects, Manson's son, Dr P. Thurburn Manson, and Mr. George Warren volunteering for the purpose. Neither of them had been abroad or previously exposed to malaria infection. The result was that infection occurred; the parasites appeared in the blood, and were associated with an attack of tertian fever.

While the malarial organisms can be examined in the living state in a fresh film of blood between slide and coverslip, finer details of structure are best made out in dried preparations stained by one of the Romanowsky methods (Appendix). When treated in this way the protoplasm of the parasite has a blue tint, while the chromatin is of a ruby red colour.

From the zoological point of view the mosquito is regarded as the definitive host of the parasite, the human subject as the intermediate host. (It is only the female mosquito which feeds on blood.) But in describing the life-history, it will be convenient to consider first the cycle in the human body, and secondly that in the mosquito. A general account of the life-history will be given to begin with and then the features of the different species will be described.

The Asexual Cycle in the Human Subject—Schizogony. With regard to this cycle (Figs. 180-182), it may be stated that the parasite is conveyed by the bite of the mosquito in the form of a small filamentous cell (measuring about 10 to 15 μ by 1 μ)—the *sporozoite*. It was generally held that the sporozoite rapidly penetrates a red corpuscle and becomes a small amœboid organism or *trophozoite*. It has been established by recent work, however, that an initial exoerythrocytic phase exists during which the parasite is multiplying within tissue cells before invading the red blood corpuscles (*vide infra*). There is then a regularly repeated asexual cycle of the parasite in the blood, the length of which cycle determines the type of the fever. During this cycle there is a growth of the trophozoites within the red corpuscles up to their complete development; schizogony then occurs. The onset of the febrile attack corresponds with the stage of schizogony and the setting free of the *merozoites*, *i.e.* with the production of a fresh generation of parasites. These soon become attached to, and probably penetrate into the interior of, the red corpuscles, becoming intra-corpuscular trophozoites, the cycle is thus completed. The parasites are most numerous in the blood during the development of the pyrexia; further, they are much more abundant in the capillaries of internal organs than in the peripheral blood. In the malignant subtertian type the process of schizogony is practically confined to the internal organs.

In addition to these forms which are part of the ordinary asexual cycle, there are derived from the trophozoites other forms, called *gametocytes*, or sexual cells, which tend to be produced especially when the infection has lasted for some time. These remain unaltered during successive attacks of pyrexia, and undergo no further change until the blood is removed from the human body. In the simple tertian (*P. vivax*) and quartan (*P. malariae*) fevers (*vide infra*) the gametocytes are rounded in form, resembling somewhat in appearance the fully developed trophozoites before schizogony, whereas in the malignant type (*P. falciparum*) they have a characteristic crescent-like, or sausage-shaped form, hence they are often spoken of as 'crescents'.

EXOERYTHROCYTIC FORMS. The observations of James and Tate

originally proved that when fowls are infected with sporozoites of *P. gallinaceum*, in addition to invasion of the red blood corpuscles, reticulo-endothelial cells in various sites become parasitised. Owing to these cells being devoid of hæmoglobin the parasites which develop in them are unpigmented. This has been confirmed for other species of bird malaria. Such exoerythrocytic forms are much less numerous when birds are inoculated with infected blood. It appears that sporozoites introduced into the blood soon become concentrated in reticulo-endothelial cells where they undergo multiplication (the primary tissue phase, which occurs during the incubation—prepatent—period) and finally invade the blood. From either the blood or the primary tissue phase a secondary tissue phase may develop later (Davey). The tissue phases of the parasite are resistant to anti-malarial drugs like quinine. In human malaria, the resistance to quinine shown by the earliest stage of natural infection conveyed by the mosquito was evidence in support of the existence of a pre-erythrocytic phase. Also, when human subjects are heavily inoculated with sporozoites of *P. vivax* or *P. falciparum* by bites of mosquitoes the parasites within a few minutes appear transiently in the blood (as shown by its infectivity for susceptible persons) and then the blood ceases to be infective till after the lapse of some days (Fairley). Recently, Shortt and his associates have demonstrated in the liver of monkeys a week after inoculation with sporozoites of *P. cynomolgi*, the occurrence of the pre-erythrocytic stage consisting of large exoerythrocytic (or pre-erythrocytic) forms; and a similar observation has been made in the case of a man heavily infected seven days previously with sporozoites of *tertian malaria*. The pre-erythrocytic forms of *P. vivax* measured up to $42\ \mu$ (Shortt, Garnham, Covell and Shute). Late relapses, such as are a feature of benign tertian infection, may likewise be due to development of the blood forms from persisting parasites of the secondary tissue phase (*vide p.* 647).

The various forms of the parasite seen in the human blood will now be described in more detail.

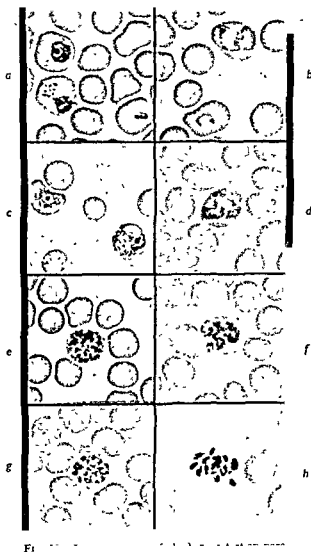
MEROZOITES are the youngest and smallest forms resulting from the segmentation of the adult amœbula or *schizont*. They are of round or oval shape and of small size, usually not exceeding $2\ \mu$ in diameter; the size, however, varies somewhat in the different species. A nucleus and peripheral protoplasm can be distinguished (Fig. 180, *h*). The former appears as a small rounded body, which usually remains unstained, but contains a minute mass of chromatin which stains a deep red with the Romanowsky method, the peripheral protoplasm being coloured fairly deeply with methylene blue. The merozoites show at first free in the plasma, they soon become encased in the trophozoites.

early stages of py merozoites, and the

TROPHOZOITES.

red corpuscles (Fig. 180, *a, b*). They usually occur singly in the red corpuscles, but sometimes two or more may be present together. As seen in fresh blood, the youngest or smallest forms are minute colourless bodies, of about the same size as the merozoites, they exhibit more or less active amœboid movement, showing marked variations in shape. The amount and character of the amœboid movement varies somewhat in different species. As they increase in size, pigment appears in their interior as minute dark brown or black specks, and gradually becomes more abundant (Fig. 180, *c, d*). This pigment, which has been identified as hæmatin, is elaborated from the hæmoglobin of the red corpuscles. The red corpuscles thus invaded may

remain unaltered in appearance (*P. malariae*, Fig. 181), or may become swollen and pale (*P. vivax*, Fig. 180). In stained specimens of the parasite the chromatin of the nucleus may be a single concentrated mass or several separate granules (coloured a deep red by the Romanowsky stain). The protoplasm of the parasite, which is coloured of varying depth of tint with methylene blue, shows great variation in configuration. The young parasites not



Fi

infrequently present a 'ring-form' due to the formation of a large vacuole, the nucleus being displaced to one side (Figs 180, *b*, 181, *b*). These ring-forms are met with in all the varieties of the parasite, but they are especially common in the case of the malignant parasite, where they are of smaller size and of more symmetrical form than in the others.

Within the red corpuscles the parasites gradually increase in size till the mature form (*schizont*) is reached (Fig. 180, *d*, *e*). In this stage the parasite loses its amœboid movement more or less completely, has a somewhat

rounded form, and contains a considerable amount of pigment. In the malignant form it only occupies a fraction of the red corpuscle (Fig. 182, *b*). The adult parasites may then undergo schizogony, but not all of them do so; some become degenerate and ultimately break down.

SCHIZONTS. In the process of schizogony the nuclear outline becomes



FIG. 181. Various phases of the malarian parasite (*Plasmodium malariae*). *a*, trophozoite showing marginal infection; *b*, trophozoite; *c*, trophozoite (quartern band); *d*, schizont; *e*, *f*, *g*, *h*, segmenting schizonts. Leishman's stain. 1,000

lost, and the chromatin divides into a number of small granules which are scattered through the protoplasm; the latter then undergoes corresponding segmentation and the small merozoites result. The pigment during the process becomes aggregated in the centre and is surrounded by a small quantity of residual protoplasm (According to Schaudinn, in the case of *P. vivax*, schizogony begins by a sort of primitive mitosis, which is then followed by simple multiple fission.) The merozoites are of rounded or oval shape and are set free by the rupture of the envelope of the red corpuscles. The pigment also becomes free and may be taken up by monocytes, the merozoites being free from pigment. The number and arrangement of the merozoites

within the schizont vary in the different species. Also, the numbers for each species show a considerable variation according to different workers; the figures given here represent the extreme ranges. In *P. malariae* there are 6 to 16, and the segmentation is in a radiate manner giving rise to the characteristic daisy-head appearance (Fig 181, g), in *P. vivax* they number 12 to

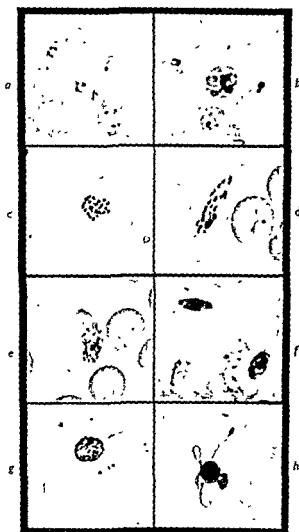


FIG 182 Various phases of the malignant malaria parasite (*Plasmodium falciparum*)

a, trophozoites in peripheral blood.

b, schizont (smeared from liver)

c, segmenting schizont (smeared from liver)

d, microgametocyte (male crescent)

e, macrogametocyte (female crescent)

f, g, h changes in form undergone by the crescents outside the body — h, microgametocyte showing exflagellation

a-c and f-h $\times 1,000$

d and e $\times 1,200$ Leishman's stain

(Figures f, g and h are from preparations lent by the late Sir Patrick Manson)

25, and have a somewhat rosette-like arrangement (Fig 180, f, g), in *P. falciparum* there are usually 8 to 32 merozoites of small size and somewhat irregularly arranged (Fig 182, c).

GAMETOCYTES These are sexual cells which are formed from certain of the trophozoites, and which undergo no further development in the human

subject. In the mild tertian and quartan fevers they are rounded and resemble somewhat the largest trophozoites (Figs. 180, 181). The female cells, *macro-gametocytes*, are relatively of large size, measuring 12 to 14 μ in diameter; they contain grains of pigment, and the protoplasm stains somewhat deeply blue with the Romanowsky stains, while the nucleus is small and compact, with deeply staining chromatin; it is commonly situated near the margin of the parasite. The male cells, *microgametocytes*, are smaller, and the protoplasm stains less deeply; the nucleus, generally in the centre, is large, with the chromatin somewhat diffuse, and often forms a broad band or spindle stretching across the cell. In first infections with *P. vivax* gametocytes appear in the peripheral blood on the seventh day of fever, and mosquitoes which ingest the blood on about the tenth day may become infected (James). In *P. falciparum* the gametocytes have the special crescentic or sausage-shaped form. They measure 9 to 14 μ by 2 to 3 μ , and occasionally a fine curved line is seen joining the extremities on the concave aspect, which represents the envelope of the red corpuscle (Fig. 182, *d, e*). They are colourless and transparent, and are enclosed by a distinct membrane. When fully developed



FIG. 183 Gametocyte of
Plasmodium vivax
Leishman's stain
 $\times 1,000$



FIG. 184 Gametocyte of
Plasmodium malariae
Leishman's stain
 $\times 1,000$

the male crescents (Fig. 182, *d*) can be distinguished from the female (Fig. 182, *e*) by their appearance. The male form has the less deeply stained protoplasm, tends to be the broader, the nuclear chromatin is diffuse, and the pigment is scattered through the cell. The female form has the more deeply stained protoplasm, is narrower, the nucleus is small and placed centrally, and the pigment is concentrated round the nucleus. The early forms of the crescents are somewhat fusiform in shape. The fully developed crescents do not appear in the blood till several days after the onset of the fever, and they may be found a considerable time after the disappearance of the pyrexial attacks; they are most numerous in cases in which the infection has lasted for some time. They are also little, if at all, influenced by the administration of quinine or atabrin, but are destroyed by plasmoquin. Ross and Thomson enumerated directly (p. 656) the malaria parasites in the blood at different stages of the disease, and found that a certain relationship exists between the asexual and the sexual forms, a rise in the number of the former being followed eight to ten days later by a rise in the number of the latter, they accordingly considered that this is probably the period necessary for the development of the sexual forms. They considered also that the long persistence of crescents in the blood after the fever has ceased, is due not to the long survival of individual crescents, but to their being constantly replenished from asexual forms which persist in the blood and pass through the ordinary process of schizogony, fever only occurring when the number of asexual forms reaches some hundreds per cubic millimetre. Crescents are most readily found in the bone marrow and spleen.

THE RELATION OF PHASES OF THE LIFE CYCLE TO RECURRENCES It is well known that after a patient has apparently recovered from malarial fever a relapse or recurrence may take place without fresh infection, sometimes many years afterwards. Several explanations have been offered which are based on features of the life cycle of the parasite. Schaudinn claimed that the macrogametocyte of tertian fever may by a process of parthenogenesis give rise to merozoites, which in their turn infect the red corpuscles and start the cycle again. It is possible also, that a recurrence coincides with the setting free in the blood stream of exoerythrocytic forms of the secondary tissue phase of the parasite. The observations of Ross and Thomson, already referred to, support the view of Bignami and others, that recurrences depend on the survival of asexual forms in small numbers, which pass through the ordinary cycle and only produce fever when they again become sufficiently numerous. Certain unusual appearances of the parasites met with in chronic infections have been interpreted as special asexual resistant forms which may survive in the blood during the latent periods of the disease. What is now known of the mechanism of immunity in malarial infections suggests that relapses are usually due to a disturbance of the equilibrium between parasite and host, owing to a lowering of the circulating antibodies of the latter, e.g. from some intercurrent illness or other so far undetermined factors (p. 653).

The Sexual Cycle in the Mosquito—Sporogony. As already explained, this starts from the gametocytes. After the blood is shed, or after it is swallowed by the mosquito, two important phenomena occur, namely (a) the full development of the sexual cells or gametocytes, and (b) the fertilisation of the female cell. The early stages in the sexual cycle can also be observed to occur *in vitro*. If the blood from a case of malignant infection be examined in a moist chamber, preferably on a warm stage, under the microscope, both male and female gametocytes may be seen to become oval and afterwards rounded in shape (Fig. 182, f, g). As Ross showed, evaporation also initiates those changes. Maturation of the female cell then takes place by the extrusion of part of the nuclear chromatin, this process corresponding to the formation of polar bodies. In the case of the male cell, a vibratile or dancing movement of the pigment granules can be seen in the interior, and soon several flagellum-like structures shoot out from the periphery (Fig. 182, h). They are of considerable length but of great fineness, and often show a somewhat bulbous extremity. By the Romanowsky method they have been found to contain a delicate filament of chromatin. They represent the male cells proper, and are known as *microgametes*. They become detached from the sphere and move away in the surrounding fluid. Fertilisation (syngamy) occurs by the fusion of a microgamete with the mature female cell (*macrogamete*), the chromatin of the two cells afterwards forming a single nucleus. Similar changes occur in the gametocytes of the other species, but, as has been said, the cells are rounded from the first. The fertilised female cell is now generally spoken of as a *zygote* or *ookinete*.

It has been established that the phenomena just described occur within the stomach of the mosquito, and that the fertilised cell or ookinete, which becomes elongated and motile, penetrates the stomach wall and settles between the muscle fibres, on the second day after the mosquito has ingested the infected blood, small rounded cells about \bar{v} to $8\ \mu$ in diameter, and containing clumps of pigment, may be found in this position. (It was, in fact, the character of the pigment which led Ross to believe that he had before him a stage in the development of the malaria parasite.) A distinct membrane forms around the ookinete, and the structure is now termed an *oöcyst*. On subsequent days a great increase in size takes place, the cysts coming to

project from the surface of the stomach into the body cavity. The contents of the oöcyst divide into
 divide and form a large m
 ment; these were called by Koss germinal rods, but are now usually known as *sporozoites*. The full development (*sporogony*) within the sporocyst occupies over seven days, the length of time depending on the temperature. When fully developed the cyst measures about $60\ \mu$ in diameter, and appears packed with sporozoites. It then bursts, and the latter are set free in the body cavity. A number settle within the large veneno-salivary gland of the insect; and are thus in a position to be injected along with its secretion into the human subject. In this way the cycle in the human subject is started, as above described. According to James, the sporozoites, to produce a successful infection, must be lying free in the common salivary duct of the mosquito at the time of biting. Daniels found that, in the case of *P. falciparum*, an interval of twelve days at least intervened between the time of feeding the mosquito and the appearance of the sporozoites in the gland
 at 24°C in a saturated atmosphere

atmosphere are both essential. There is no evidence that the malarial parasites in the mosquitoes pass to the next generation in the eggs. An infected mosquito may rid itself of sporozoites by repeated biting, apart from this, when once infected it remains infective for long periods, e.g. three months in the case of the benign tertian parasite; also the infection may persist over the winter when the insect hibernates (James). However, the sporozoites in the mosquito may finally become degenerate and so cease to be infective for man.

It will thus be seen that in the human subject the parasite passes through an indefinite number of regularly recurring asexual cycles, with production of collateral sexual cells, and that in the mosquito there is one cycle which may be said to start with the fertilisation of the female gamete.

Species of the Malaria Parasites. Laveran's original view was that there is only one species of malaria parasite, which is polymorphous, and presents slight differences in structural character in the different types of fever. It is now generally accepted that there are at least three distinct species which infect the human subject. Practically all are agreed as to a division into two groups, one of which embraces the parasites of the milder fevers—'winter-spring' fevers of Italian writers—there being in this group two distinct species, for the quartan and tertian types respectively; whilst the other includes the parasite of the severer forms—'æstivo-autumnal' fevers, malignant or pernicious fevers of the tropics, or irregularly remittent or sub-tertian fevers. Formerly Italian writers distinguished several varieties of the latter parasite, though the morphological differences described were slight. Further observations, however, threw doubt on this distinction, and the evidence tended to show that there is a single species. Opinion also varied as to the cycle of this parasite: according to some observers, twenty-four hours, according to others forty-eight hours, though there is more evidence in support of the latter view, and the term 'malignant tertian' or 'sub-tertian' is frequently used. The fever is often of a prolonged and irregular type and multiple infection is probably common. We may therefore speak of three well-defined species of the malaria parasite of man: *Plasmodium vivax* of benign tertian malaria, *Plasmodium malariae* of quartan malaria, and *Plasmodium falciparum* (*Laverania malariae*) of malignant and sub-tertian malaria. In addition *Plasmodium ovale* probably constitutes a fourth species.

while man can be infected experimentally with *Plasmodium knowlesi*, which occurs naturally in monkeys.

P. vivax—the Parasite of Benign Tertian Fever. The cycle of development is completed in forty-eight hours. The trophozoites have a less refractile margin than in the quartan type, and are thus less easily distinguished in the fresh blood; the amoeboid movements are, however, much more active, while longer and more slender processes are given off. The infected corpuscles become enlarged and pale, and when stained by a Romanowsky method tend to show numerous round dots coloured mauve to rose—'Schuffner's dots'. The pigment within the parasite is fine and of yellowish-brown tint. The mature schizont is rather larger than in the quartan, has a rosette appearance, and gives rise to from twelve to twenty-five merozoites, though sometimes even more occur, these have a somewhat oval shape (Fig. 180, g, h). All the stages of development occur in the peripheral blood.

When the blood is examined parasites of the benign tertian variety may be found simultaneously in several stages of development, and in such cases the fever is of a quotidian type. It was believed that this pointed to a double infection, but the observations of James indicate another possible explanation. Patients were investigated whose primary attack of malaria was caused by a single bite of one mosquito infected with a pure strain of *P. vivax* under experimental conditions. It was found that the 'initial' stage of infection, lasting two to five days, was characterised by gradually increasing irregular fever without rigors. This was followed in 80 per cent of cases by the 'developed' stage with quotidian fever and rigors, which often lasted more than ten days. The 'terminal' stage then set in, the quotidian fever changing to that of tertian type and the severity of the paroxysms gradually diminishing. Examination of the peripheral blood throughout these stages showed that soon after the first appearance of the parasites different phases of growth are found together—an occurrence which is explained by sporozoites received from the mosquito not being all equal in their rate of development. Then in the developed stage two groups become dominant and determine the quotidian fever. Finally, the tertian type of attack is due to impending recovery, one of the two groups of parasites having disappeared before the other.

P. malarie—the Parasite of Quartan Fever. The cycle of development in man is seventy-two hours, and produces pyrexia every third day; double or triple infection may, however, occur. In fresh specimens of blood the outline is more distinct than that of the tertian parasite, and amoeboid movement is less marked. Only the smaller forms show movement, and this is not of active character. The infected red corpuscles do not become altered in size or general appearance, but by prolonged staining a stippling ('Ziemann's stippling') may be demonstrated—dots and points coloured like Schuffner's dots, but smaller and less distinct. The pigment within the parasite is in the form of coarse granules, of dark brown or almost black colour. Medium-sized parasites may appear as band-shaped forms in the corpuscles (Fig. 181, c). The fully developed schizont has a 'daisy-head' appearance, dividing by regular radial segmentation into from six to sixteen merozoites, which, on becoming free, are rounded in form (Fig. 181, e, f, g, h). As in the case of *P. vivax*, all the stages of development can be readily observed in the peripheral blood. The gametocytes have a rounded form as described above.

P. falciparum or *Laverania malarie*—the Parasite of Malignant or Sub-Tertian Fever. The cycle in the human subject probably occupies forty-eight hours, though this cannot be definitely stated to be always the case (*vide supra*). In the peripheral blood the trophozoites in the red corpuscles are of

small size, and their amœboid movements are very active; they often, however, pass into the quiescent ring form (Fig. 182, a). The young forms are often found at the edge of the red cells and are elongated ovals or streaks, the rings frequently project from the margin of the corpuscle. The pigment granules, even in the larger forms, are few in number and very fine. The infected red corpuscles may be unaltered or tend to shrivel and assume a deeper or coppery tint, sometimes they are swollen and decolorised. They may contain 'Maurer's spots', which are scantier, larger, and more irregular than Schuffner's dots; they stain mauve to violet. The proportion of infected red corpuscles is much larger in the internal organs. Schizogony takes place almost exclusively in the internal organs—spleen, etc. Usually no schizonts can be found in the blood taken in the usual way, but they may be observed in the very severe or pernicious type of the disease. The fully developed schizont usually occupies less than half the red corpuscle, and produces from eight to thirty-two merozoites, somewhat irregularly arranged and of minute size (Fig. 182, c). The gametocytes, which occur in the peripheral blood, have the crescentic form, as already described.

Cases of infection with the malignant parasite tend to assume a pernicious character, and then the number of organisms in the interior of the body may be enormous. In certain fatal cases with coma the cerebral capillaries appear to be almost filled with them, many parasites being in process of schizogony; and in so-called algid cases, characterised by great collapse, a similar condition has been found in the capillaries of the omentum and intestines. The blockage of capillaries, to which the serious effects are in great part attributed, is due to the infected red cells in this variety of malaria becoming agglutinated. There are various other features of the pernicious type, e.g. icterus, ante- and neo-natal mortality. The process of blood destruction, present in all malarial fevers, reaches its maximum in the malignant class, and the brown or black pigment elaborated by the parasites—in part after being taken up by leucocytes, chiefly the macrophages—becomes deposited in various organs, spleen, liver, brain, etc., especially in the endothelium of vessels and the perivascular lymphatics. In the severer forms also brownish-yellow pigment is apparently derived from liberated hæmoglobin, and accumulates in various parts, especially in the liver cells, most of this latter gives the reaction of hæmosiderin.

Plasmodium ovale has been reported (Stephens). The fever is of the tertian kind which shows some similarity to *P. falciparum*. The pigment is lighter in colour and less coarse. The red cells are slightly enlarged and often exhibit frayed or fimbriated edges. Medium-sized trophozoites assume an oval shape and the red cells also are oval. A pronounced feature of the blood corpuscles is their granular stippling likened to the Schüffner's dots of benign tertian malaria, but the granules are more distinct, coarser, and stain more intensely. The schizont yields 6 to 12 merozoites. The sexual cycle of this parasite has been studied in *Anopheles maculipennis*. The infection produced experimentally in general paralytics is very mild and becomes cured spontaneously.

Plasmodium knowlesi. This parasite occurs naturally as a mild infection of the Malayan monkey, *Macacus irus*, but when transmitted to *Macacus rhesus* produces a severe and rapidly fatal infection. Its pathogenicity on experimental inoculation in man has been demonstrated by Knowles and Das Gupta. The parasite resembles the other *Plasmodia* in general characters. Typical ring forms with one or two chromatin dots are a common feature of blood films. Band forms of the parasite in red cells (cf. *P. malariae*)

are frequently observed. The pigment granules of the parasite tend to be large and dark-coloured (Fig. 185). The red cells are not usually enlarged, but a fully developed parasite may occupy practically the whole erythrocyte. Schizonts divide irregularly into about 10 to 12 merozoites. The gametocytes are rounded in shape like those of *P. vivax* and *P. malariae*. The morphology tends to vary in different genera of monkeys. Van Kooyen and Pale have recorded successful therapeutic results with it in general paralysis. The infection can easily be maintained in *M. rhesus* monkeys in the laboratory and the human infection can be induced by intravenous or intramuscular injection of defibrinated blood from these animals. Moreover, such blood when kept at 0° C. retains its infectivity for the human subject for eight days and for sixteen days in the case of the monkeys. In man the incubation period is usually eight days. The resulting fever is of quotidian type. The infection can be immediately terminated when necessary by a single intramuscular injection of quinine (10 grains), though atabrin may be ineffective, but early recovery tends to occur spontaneously.

Cultivation of Malaria Plasmodia. Bass and Johns obtained growths of the parasites of tertian and malignant fevers outside the body. To defibrinated blood from malarial patients 1 per cent. of a 50 per cent. solution of dextrose in water was added. Growth of the parasites took place within the red corpuscles, but only under anaerobic conditions, so that a layer of serum at least half an inch in depth above the sedimented corpuscles was necessary. Under such circumstances, the parasites underwent enlargement and afterwards passed through the stage of schizogony. The merozoites after becoming free are destroyed by leucocytes but if measures are taken to prevent the presence of these other generations of growth may be obtained in similarly prepared tubes of blood with sufficient serum. (According to J. G. and D. Thomson, removal of the leucocytes is unnecessary.) The parasites flourish only in the superficial layers of the sedimented corpuscles, and the most suitable temperature is 37 to 41° C. Thomson and MacLellan found that in cultures the malignant parasites show a tendency to clump before and during the stage of sporulation, on the other hand the benign tertian parasites do not clump. Cultures tend to die out after a few passages.



FIG. 185. *Plasmodium knowlesi* (parasite of monkey malaria) in a film of blood from a case of general paralysis under malaria therapy. Fully grown trophozoites in corpuscles which are of normal size. Note the coarse granules. Leishman's stain. $\times 1,122$. (From a photograph by Prof. C. L. van Kooyen.)

Recently German, Anfinson and others have defined more precisely the conditions required for the proliferation *in vitro* of *P. knowlesi*. The culture medium consisted of a mixture of inorganic salts corresponding with that in monkey plasma, along with organic compounds including glucose, pantothenic acid, amino acids, etc. Substances of the class of water-soluble vitamins (ascorbic acid, folic acid, etc.) also proteins and pyrimidines. In this in a special form of flask were added suitable proportions of normal monkey blood and 1 parasitized red cell obtained by differential sedimentation of infected blood to form a shallow layer. The flask was kept at 36 to 38° C. and rocked slowly, while a flow was kept up of a mixture of air with 5 per cent. CO_2 at 11 to 14 mm. Hg was maintained. In 24 hours, which is the period of the asexual cycle of the parasite, the number of parasites was 2,344 (45). Such cultures were successful when the fluid medium was enriched with whole blood or serum. A mixture of the parasites was preserved as a monkey inoculated from the 7th culture developed a severe infection. Hawking has obtained cultures of the

exoerythrocytic forms of *P. gallinaceum* in cultures *in vitro* of chicken tissue containing macrophages and reticulo-endothelial cells. *P. relictum* has been cultivated similarly.

Pathology. While much work has been done on the malaria parasite, relatively less attention has been directed to the processes by which it pro-

associated with particular stages in the life cycle. Thus the pyrexia occurs when the stage of schizogony is actively in progress, but nothing definite is known as to its real nature or mode of production—whether it is due to a toxin or is allergic in character. It should be noted in this connection that an individual whose blood is swarming with the parasites may be free from attacks of fever. We can better explain the anæmia which is so pronounced in cases where the disease is of long standing, and which is due to the actual destruction of red blood corpuscles. The parasite in its sojourn in these cells absorbs their pigment and thus destroys their function; this is further indicated by the activity displayed by the red marrow in its attempts to make



FIG 186 Mononuclear leucocyte from blood in a case of malaria (quartan) showing ingested particles of pigment. Leishman's stain $\times 1,000$.

good the loss sustained by the blood. One of the most interesting events in malaria, and one that links it with bacterial infections, is the reaction of the leucocytes. It has been shown that during the apyrexial stages the total number of leucocytes varies greatly, but that there is always an increase of the mononuclear cells, these frequently numbering 20 per cent. or more of the whole, and sometimes even outnumbering the polymorphs. The presence of pigment in the mononuclear leucocytes (due to phagocytosis of pigmented parasites and pigment liberated after schizogony) is an important feature, since in cases where the parasites themselves cannot be demonstrated in the blood it may be accepted as conclusive evidence that the case is

really one of malaria (Fig. 186). Other protozoal diseases resemble malaria in this activity of the mononuclear cells. The macrophages in the spleen, liver, and bone marrow play an active part in the destruction of the parasites (see Taliaferro *et al.*). Enlargement of the spleen is a marked feature, especially in chronic malaria; its prevalence in native children affords a rough indication of the incidence of the infection in regions where the disease is endemic.

Blackwater Fever. Knowledge regarding the relation of blackwater fever to malaria is unsatisfactory. Blackwater fever occurs, especially in Europeans, in tropical countries where malaria exists, and particularly after repeated attacks of the malignant type contracted in West Africa (see also Thomson's investigations in Rhodesia). It is characterised by pyrexia, jaundice, darkly-coloured urine—the colour being due to altered hæmoglobin pigment—delirium and collapse, and anuria frequently ending in coma and death. By some the condition has been looked on as a separate disease, by

others as a complication of malaria. With regard to the former view, Stephens has stated that in the great majority of cases there is direct or indirect evidence of repeated attacks of malaria; that while in all cases there must be an agent at work causing hæmolysis, in some there is the possibility of that agent being quinine. The essential feature is an extracellular destruction of red corpuscles in the

blood plasma (lysæmia). According to Christophers and Bentley this is not directly due to parasitic, osmotic, or chemical actions, but to a specific hæmolysin arising in the body as the result of the repeated blood destruction. But the presence of such lysin in the serum has not been established; the existence of an intracellular lytic enzyme has been suggested (Fairley *et al.*) The occurrence of lysæmia may be precipitated by an acute attack of malaria especially when under certain circumstances this is associated with the administration of quinine. It still remains to be determined, however, what other factors are responsible for the onset of an attack of blackwater fever. According to the views of Fairley and Bromfield the hæmolytic substance arises in chronic malignant malaria from some cellular metabolic breakdown precipitated by quinine, chill, etc. Its action is to lyse the corpuscles, and oxidised hæmatin liberated from the hæmoglobin combines to form methæmalbumin which can be demonstrated in the plasma (Fairley). According to Gear, the onset of an attack depends on the sudden discharge into the general circulation of a large amount of hæmolysin accumulated in the spleen. Maegraith *et al.* have found that sera from patients with blackwater fever are deficient in the property of inhibiting the lysis of red cells by human tissues, which normal sera possess. They suggest that this disturbance of balance may explain the lysæmia in blackwater cases.

Immunity. The question of immunity to malaria is one of great importance. In such a malaria-stricken region as the West Coast of Africa there can be little doubt that malaria in the negro is a much less common disease than in the European. Koch from his observations on the natives of the West Coast to the infection of the natives in the adult community. Stephens and Christophers have shown that in children harboured malaria the presence of parasites in children might appear to preclude the possibility that the immunity of the adult is due to survival of the most resistant, but the infant mortality in these regions may be very high, and such a survival may be the real explanation. Koch stated also that although an immunity appears to exist in native adults in malarial districts, this is only true of those born in the locality—natives coming from neighbouring non-malarial districts into the malarial region being liable to contract the disease. It would appear that for the maintenance of immunity frequently repeated reinfection (superinfection) is required. But of the existence of acquired immunity there can be no doubt, since a person who has suffered from an experimentally induced malarial attack, especially when spontaneous recovery from a relapse has occurred, is immune to reinfection. Such immunity is not merely species-specific, but is strain-specific. Some individuals, however, are naturally resistant to malarial infection as tested by experimental inoculation (James).

Recent experimental work of Coggeshall and others on malaria of monkeys has shown that the mechanism of immunity to the plasmodia is similar to that in the case of most other pathogenic organisms, i.e. antibodies are concerned. *P. knowlesi* is highly virulent for rhesus monkeys, the parasites appearing in very great numbers in the blood after inoculation and death almost invariably following. Treatment with quinine renders the infection chronic, but does not produce sterilisation, as parasites are found in fluctuating numbers in the blood for months or years afterwards. (If splenectomy is performed on such monkeys an acute recurrence develops.) At the chronic stage, if a large number of plasmodia of the same strain are injected into the circulation, they disappear in a few hours and there is no disturbance of health, i.e. there is immunity to superinfection. Also, the serum of the chronically infected

animal contains specific protective antibodies; thus a susceptible monkey inoculated with a mixture of such immune serum and parasites fails to become infected or acquires a *non-fatal infection*. (Similar antibodies are present in the serum of men chronically infected.) For the demonstration of protective antibodies it is essential not to use an excessive inoculum of the parasites; failure to observe this precaution probably explains the inability to detect antibodies which has been reported. In the course of a chronic infection the level of antibody in the blood varies, a marked fall being followed by a relapse, after which the antibody rises again, even although parasites persist in the blood. Immunity developed by a chronic infection lasts for *many months after complete cure*, which can be brought about by treatment with a sulphonamide drug. The antibody is removed from immune serum *in vitro* by contact with the plasmodia, although the latter undergo no obvious morphological changes. The parasites are most susceptible to antiserum when mature, just before sporulation. This may be due to the envelope of the red blood corpuscles being damaged at this stage, thus rendering the organisms more accessible to the antibody. Other evidences of antibody action can also be elicited *in vitro*, *viz.* the complement-fixation reaction and

malaria. On the other hand, the agglutinins, like the protective antibodies, are species-specific.

Immunity does not follow treatment of the primary acute infection, even although very large numbers of parasites are destroyed in the body. Similarly, the injection of vaccines of dead organisms is ineffective. It appears that a prolonged action of the antigens is necessary for immunisation. Recently it has been claimed that the use of 'adjuvants', such as liquid paraffin or killed tubercle bacilli, along with the dead plasmodia leads to immunity to the latter (Freund *et al.*).

General Considerations. The development of the malaria parasites in the mosquito and infection of the human subject through the bites of this insect, as well as the evidence that none of the lower animals takes the place of man as intermediate host, point to several definite methods of prevention of infection, which have to a certain extent already been practically tested. These depend essentially on destruction of the mosquitoes or protection of man from their bites. Extensive observations go to show that all the mosquitoes which act as hosts of the parasite belong to the genus *Anopheles*; of these, there are a large number of species, and there are good grounds for accepting about sixty of them as transmitters of the disease under natural conditions. Some of these anophelids occur in Great Britain, especially in regions where malaria formerly prevailed. The opportunity for infection from cases of malaria returning from the tropics to this country thus exists, and such infection has occurred. Destruction of mosquitoes may be effected by removal of their breeding places. Many species lay their eggs in stagnant pools and other collections of standing water. Accordingly the removal, where practicable, by filling in or drainage of such collections in the vicinity of centres of population, the covering in of wells, etc., may be effective. The killing of the larvæ may be brought about by spraying or sprinkling on the water, petroleum, pyrethrum, or a mixture of Paris green (arsenite) with a suitable dust (often by means of an aeroplane); these measures must be repeated at short intervals—ten to fourteen days. Such procedures have been carried out in various places with marked success. Also in more open waters fish (*Gambusia*, *Tilapia*) have been used for the control of mosquito

larvæ. Adult mosquitoes may be killed in houses by spraying with insecticides, or diverted to animals in the vicinity, such as pigs. Recently spraying with emulsions or oily solutions of 'D D T' has been adopted against both the mosquitoes and their larvæ. For antimalarial measures an intimate knowledge of all the local conditions is essential. For instance, in North Holland epidemic foci of benign tertian malaria have been traced to the capacity of one race (*atropareus*) of *A. maculipennis*—which behaves as a species—to breed in brackish water left by the draining of the Zuyder Zee. This race of the mosquito also continues to take meals of blood after the egg-laying period is past, i.e. at a time when its length of life is greater than earlier in the season. Therefore its chances of conveying infection are much increased (Swellingrebel and de Buck). The 'species eradication' of *A. gambia* carried out in Brazil by the Rockefeller Foundation along with the Government shows what can be accomplished by thorough application of the above methods (Soper and Wilson). The other method of control is the protection against mosquito bites by netting, it being fortunately the habit of the anopheles rarely to become active before sundown. The experiments of Sambon and Low in the Campagna proved that individuals using this means of protection may live in a highly malarial district without becoming infected. In the tropics the natives in large proportion suffer from malarial infection, but often, having acquired immunity in childhood, act as carriers. One would accordingly expect that infection of the mosquitoes in the neighbourhood of native settlements would be common. This has been found to be actually the case, and the dwellings of Europeans should as far as possible be at some distance from the native centres of population. The administration of antimalarial drugs to persons living in highly malarial regions, in order to prevent as well as to treat infection, has also been recommended. There appears to be general agreement that in India the properly controlled administration of such drugs (p. 707) must, in the meantime at least, be the chief means of combating the disease.

Different strains of the same species of malaria parasite may differ in virulence. Thus the North-Holland strain of *P. vivax* when transmitted by mosquitoes causes infections which frequently do not become active until a latent period of some months has elapsed, whereas the 'Madagascar' strain of James, under similar conditions, has an incubation period of under three weeks usually, the mean being two weeks. Another feature is that certain cases are 'good infectors' of mosquitoes in contrast to others who are 'bad infectors'. This property seems to be largely independent of the number of gametocytes present in the blood or the proportion of male to female forms. Individual mosquitoes of the same species under similar conditions differ also in their tendency to produce mature zygotes.

It should also be mentioned, although not bearing on the natural modes of infection, that the disease can be communicated from one person to another by injecting blood containing the parasites. Usually $\frac{1}{2}$ to 1 c.c. of blood is infective, but the result is more certain in intravenous than in subcutaneous injection. In such cases there is an incubation period, usually of from seven to fourteen days, after which the fever occurs, the same type of fever is reproduced as was present in the patient from whom the blood was taken. This method of inoculation as well as that by the bites of infected mosquitoes, has been applied in recent years for the treatment of general paralysis, and it has been shown that malarial attacks produced in this way exert a beneficial effect in the disease. The question has not yet been settled whether this therapeutic effect is due entirely to the high temperature produced.

case a tick. The cycle of development of *B. bigemina* in the tick is as follows (Dennis). Certain of the piroplasms in the red corpuscles are gametocytes. When these are taken into the alimentary tract of the tick they develop into elongated motile bodies, the gametes (which distinguish the sexes). Fusion of two gametes passes through the thin wall of the gut and penetrates the body wall of the tick.

The cy-

kinetes

the tris-

sporoblasts

plasm form, pass out from the salivary glands in the saliva, so infecting a fresh host bitten by the tick. Accordingly the infection passes on to the next generation.

of piroplasmosis it is immune

cases very interesting facts have been observed. For instance, the condition may not be associated with the disappearance of the parasite from the blood of the immune animal, and the latter may thus be a source of danger to other non-immune animals with which ticks harboured by it may come in contact.

The following are some of the chief piroplasms causing disease in animals.

Babesia bigemina gets its name from the fact that it is often present in the red cells in pairs, which may be attached to one another by a fine thread of protoplasm, this probably results from the complete separation of two individuals being delayed after division has occurred. It is the largest of the piroplasms found in cattle. Infection is spread by the tick *Boophilus bovis* (*Margaropus annulatus*), and some of the characteristics of the disease epidemiologically are explained by the fact that this insect goes through all its moultings on the same individual host, but other ticks have also been shown to spread the infection. *Babesia bovis* is also a cause of red water in cattle, it is much smaller than *B. bigemina*. The tick *Ixodes ricinus* transmits the infection. *Babesia equi* give rise to biliary fever in horses in South Africa. As was shown by Theiler, it is carried by the tick *Rhipicephalus evertsi*. Mules and donkeys can also be infected. Young horses are less severely affected than older ones. After clinical recovery the blood of such 'salted' animals can be used for the protective inoculation of young horses. *Babesia canis* causes a piroplasmosis occurring in dogs. The organism has been cultivated by Thomson and Fantham and others by the method of Bass and Johns (p. 651).

Theileria parva. This organism was discovered by Theiler in the blood of cattle suffering from African East Coast fever, a serious disease characterised by fever and enlargement of the lymphatic glands, but without the occurrence of hæmoglobinuria. The organism is small, appearing as ovoid or rod-shaped forms which measure 1 to 3 μ in length and 0.5 to 0.7 μ in breadth. *Rhipicephalus appendiculatus* and *evertsi* and certain other ticks convey the disease. It is of interest that in the case of this organism as contrasted with the piroplasms described above, division does not occur in the red corpuscles but in endothelial cells of the lymphatic glands, spleen, and other organs. The schizonts appear as masses of protoplasm 3 to 10 μ in diameter, containing a large number of minute chromatin dots. The intracellular forms of the parasite appear to be adapted for development in the tick, since inoculation of blood from an infected animal into other cattle does not produce the disease.

With regard to the pathology of infection by piroplasms and *Theileria* nothing is known, apart from the fact that certain of them cause destruction of the red blood corpuscles. The diseases are often extremely fatal, carrying off nearly every individual attacked.

CHAPTER XXXIV

PATHOGENIC PROTOZOA (contd.)—ENTAMŒBA HISTOLYTICA AND COMMENSAL AMŒBÆ FLAGELLATES OF THE ALIMENTARY AND GENITO-URINARY TRACTS · BALANTIDIUM COLI

ENTAMŒBA HISTOLYTICA OF TROPICAL DYSENTERY

In a previous chapter it has been pointed out that the term 'dysentery' has been applied to conditions of different etiology, and the relations of bacteria as causal agents have been discussed (*vide* p. 352). We shall consider here that variety of dysentery which is due to an amœba and hence often known as *amœbic dysentery* or tropical dysentery, since it occurs especially in tropical or subtropical regions.

Among the early researches on the relation of organisms to dysentery probably the most important are those of Losch, who noted the presence and described the characters of amœbæ in the stools of a person suffering from the disease, and considered that they were probably the causal agents. Further observations on a more extended scale were made by Kartulis with confirmatory results, this observer finding the same organisms also in liver abscesses associated with dysentery. Councilman and Lafleur, working in Baltimore, showed that this variety of dysentery can be distinguished from other forms, not only by the presence of amœbæ, but also by its pathological anatomy. The intestinal lesions, to which reference is made below, are of a grave character, and attended with considerable mortality, also, there is a great tendency for the infection to be protracted, with the development of extensive tissue changes. A further serious feature is the production of lesions in solid organs, especially the liver ('amœbic abscess'), by amœbæ which have been carried in the circulation. The subject of the causal organism was complicated by the fact that a somewhat similar organism—'*Amœba coli*'—had been previously found in the intestine in normal conditions and in other diseases than dysentery (by Cunningham and Lewis *et al.*, and additional research confirmed these results. The characters of the common commensal amœba of the colon and the amœba of dysentery were worked out by Schaudinn, who recognised them to be distinct species, to which he gave the names of *Entamoeba coli* and *Entamoeba histolytica* respectively. Huber afterwards described an entamœba of dysentery, which in the encysted stage contained four nuclei. Viereck confirmed these observations and gave the name '*Entamoeba tetragena*' to the organism. This organ-

Further research has resulted in the discovery that *E. histolytica* and *E. tetragena* are the same organism, and *E. histolytica* is being retained, but its process of encystment corresponds with that originally described in the case of *E. tetragena*. Moreover, a small entamœba, described by Elmassian under the name '*E. minuta*', is also now known to represent merely a stage in the life-history of *E. histolytica*. Within recent years several other species of intestinal amœbæ have been identified. These, like the *E. coli*, are non-pathogenic commensals (*vide infra*). In the mouth *Entamoeba gingivalis* occurs as a commensal.

MICROSCOPIC CHARACTERS. As seen in the stools of acute dysentery, it is present in the form of rounded, oval, or pear-shaped cells, the rounded cells measuring 15 to 50 μ in diameter, the average being 20 to 25 μ (Figs. 187, 188, 189). When at rest, a clear, highly refractile ectoplasm and a granular or sometimes vacuolated endoplasm may sometimes be distinguished,

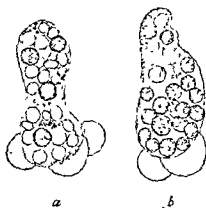


FIG 187 *Entamoeba histolytica*.

a and b, amœbæ as seen in stools, showing blunt amœboid processes of ectoplasm. The endoplasm of a shows a nucleus, three red corpuscles, and numerous vacuoles; that of b, numerous red corpuscles and a few vacuoles. Fresh specimens $\times 650$ (ca).

though this is not always the case. The nucleus is rounded, as a rule about 7 μ in diameter, and is seen with difficulty; its position is usually excentric, and is sometimes quite at the margin of the endoplasm. In stained specimens it is seen to be poor in chromatin, which is arranged as small granules under the nuclear membrane; a small chromatic karyosome is present in the centre of the nucleus (Fig. 189, A, B, C).

In perfectly fresh material very active amœboid movements are seen, which are of a flowing type. When the activity of the organism lessens pseudopodia may be suddenly protruded and later retracted, and these may consist almost entirely of ectoplasm. Those appearances when present are characteristic and of great service in the identification of the organism. The amœboid

movements may not lead to much change in position. The ingestion of red corpuscles, though not always present, is a characteristic feature of the organism, and thus of special importance in its recognition; a considerable number of red corpuscles may be contained in an amœba. The amœbæ usually die and undergo disintegration in a comparatively short time after being removed from the body, the stools ought therefore to be examined in as fresh a state as possible. According to Dobell *E. histolytica* does not ingest bacteria, therefore the presence of bacteria within the organism indicates an invasion of a degenerate amœba, or, occasionally, a true parasitism by the bacteria.

Multiplication of the amœbæ occurs by division into two equal cells, but accounts given of the process vary. Appearances of division are rarely seen in the stools, and the process occurs mainly, if not exclusively, in the lesions. Dobell, from a study of cultures chiefly, regards the process as exclusively one of mitotic division. Thus the life-history is completely asexual.

Cysts. As the symptoms of the disease abate, the entamœbæ undergo certain changes which ultimately result in their encystment. The cysts, when formed, are

The cyst wall is thin, with double contour, and sometimes only two, nuclei can be seen in the fresh condition (the presence of more than four nuclei in cysts of *E. histolytica* is very uncommon). In fixed and

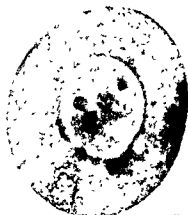


FIG 189 *Entamoeba histolytica* in film from liver. Stained with iron haematoxylin $\times 750$.

in diameter—although in different strains the cyst four, or sometimes more than four, nuclei can be seen in the fresh condition (the presence of more than four nuclei in cysts of *E. histolytica* is very uncommon). In fixed and

stained specimens the nuclei have the chromatin at the periphery with a small central karyosome, as in the active amœbæ (Fig 190, J-P). Beside the nuclei one or more elongated 'chromatoid' bodies may be seen, and there is also a vacuole (or several) containing glycogen, but no other inclusions. In the transition from the active amœboid form to the cystic stage the

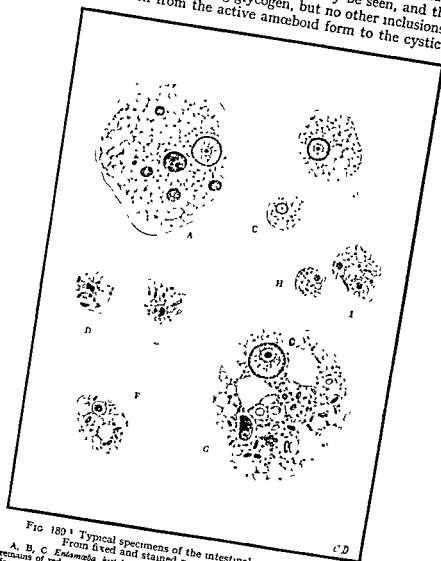


FIG 180. Typical specimens of the intestinal amœbæ of man. From fixed and stained preparations $\times 1,500$. A, B, C *Entamoeba histolytica*. (A) Large tissue-inhabiting form, containing remains of red corpuscles. (B) Pre-cystic form, belonging to a race forming cysts usually 12 μ to 14 μ in diameter. (C) Pre-cystic amœbæ of a race forming cysts usually 7 to 8 μ in diameter. D, E *Endolimax nana*. F *Entamoeba disparilis*. G *Entamoeba coli*. H, I *Dielamœba fragilis*—uninucleate and typical binucleate individuals respectively.

following changes occur. The amœba becomes smaller and the nucleus more distinct and richer in chromatin, though still maintaining its characteristic features. Further diminution in size occurs, probably by division, the small cell loses its amœboid property, and a hyaline cyst-wall forms around it.

¹ We are indebted to Colonel Byam and Dr Archibald, and to Mr Clifford Dobell for permission to use four figs which are reproduced from *The Practice of Medicine in the Tropics*.

The nucleus then divides into two or into four, as the case may be. The transition forms are to be met with in stools which are losing the typically dysenteric character. Cysts constitute the resistant and resting stage of the parasite. It is important to recognise that they are not present in acute

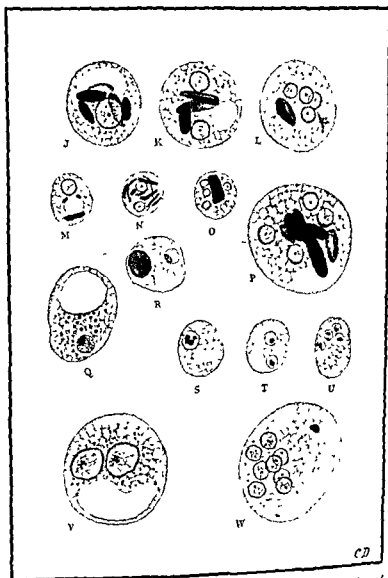


FIG. 190. Typical specimens of the cysts of the intestinal amoeba of man. From fixed and stained preparations $\times 1,500$

..... nucleate and quadrinucleate
..... μ in
..... μ in
..... μ in

in balsam)

cases, but in convalescents and carriers. They occur only in the intestine, and are never present in the secondary abscesses. The cysts continue to be found in the faeces for a long time, and they have been detected several years after dysenteric symptoms have disappeared. It is considered by some that they may persist during the rest of the patient's life. Both encystation and the development of vegetative forms from cysts depend largely on the accompanying bacterial flora (Dobell *et al*). As the cysts are the chief, if not

exclusive, means of infecting other persons, the individuals passing them are to be regarded as carriers dangerous to the community, such individuals are known as 'convalescent carriers'. In connection with the treatment of amœbic dysentery by emetine, it is to be noted that failure to cure in some cases cannot be ascribed to resistance to the drug on the part of cysts which may be present.

VIABILITY As has been noted above, outside the body the vegetative forms of *E. histolytica* rapidly die and disintegrate. Unlike the latter, the cysts have considerable powers of resistance, and in the moist condition, when kept cool, may survive for several weeks outside the body. Drying, however, rapidly kills the cysts. Unlike the vegetative forms, too, the cysts resist the acid of the gastric juice. Therefore it is by swallowing material containing cysts that the infection is spread naturally.

CULTIVATION Cultures of amœbæ in association with various bacteria were obtained by several workers, e.g. Lesage, Musgrave and Clegg, Noc and others, from cases of dysentery and also from various sources outside the body, but in no instance could the amœba grown be established as identical with *E. histolytica*. In 1925, however, Boeck and Drbohlav published an account of successful cultivation of *E. histolytica* on a medium composed of Locke's solution, egg, and serum or albumin (*vide infra*), and these results have been fully confirmed. Growth was obtained by inoculating the medium with material from dysentery stools, and subcultures, made by transferring some fluid from the deep part of the culture tube by means of a glass pipette have been maintained for many years. The amœbæ in cultures were found to be comparatively short-lived, dying out after four or five days, and subcultures were made every two days.

lesions in kittens on injection *per rectum* in eleven out of sixteen animals. These, liver abscesses also developed. The inoculation experiments were carried on over a period of five months of subculturing, and there was no evidence that the amœbæ became diminished in virulence. Dobell and Laidlaw, however, found that on subculturing loss of virulence might finally occur. It appears that a distinction should be made between pathogenicity and infectivity. Infectivity declines when a strain ceases to produce cysts in cultures (see Chang). The formation of cysts in cultures on Boeck and Drbohlav's medium occurred only rarely. Dobell and Laidlaw found, however, that the addition of sterile solid rice-starch to the medium of Boeck and Drbohlav gave more luxurious and prolonged growth of the amœba, and also enabled the whole life cycle, including excystation from the cysts, to be observed *in vitro*. Encystment depends also on the bacterial flora of cultures

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until the mixture has solidified. They are then transferred to the autoclave and sterilised (120° C for twenty minutes). The medium in each tube is covered to a depth of 1 c.c. above the slant with a mixture composed of eight parts of sterile Locke's solution and one part of sterile inactivated human blood serum. The tubes are then incubated to determine sterility. In place of the human serum a solution of crystallised egg albumin may be substituted. A 1 per cent solution of the albumin in Locke's solution is prepared and sterilised by passing through a Berkefeld filter. It is then added to the tubes containing the egg slants as above described. The initial reaction of these media varies from pH 7.2 to 7.8 and requires no adjustment. Material containing the amœbæ is added with a sterile capillary pipette to the previously warmed medium and the culture is

incubated at 37° C. The amœbæ are usually most abundant on the second day, being

(a mixture of the whites of four eggs with 1 lit sterilised by passing through a Seitz filter) or

Ringer's solution and filtered. Before inoculation, by means of a platinum loop or spatula, a small amount of rice starch is added, which previously has been sterilised in small test-tubes each containing about 2.5 grms, by dry heat (180° C. for one hour)

(B) This medium is similar except that the slope consists of undiluted horse serum at 37° C. for sixty to seventy days. The finished medium is

again tested for sterility at 37° C. Starch is added just before use. In this medium, *E. histolytica* lives for at least a week.

Cultures free from living bacteria have recently been obtained by means of penicillin (Jacobs)

Distribution and Pathological Effects. As already stated, the organisms are usually found in large numbers in the contents of the large intestine in amœbic dysentery. Their real habitat, however, is the tissues, where they exert a well-marked action. The amœbæ appear to produce a digestive softening of the tissues, hence the term 'histolytica' is an appropriate one. The lesions are chiefly in the large intestine, especially in the cæcum and rectum, and at the flexures, though they may also be present in the lower part of the ileum. At first there are local swellings on the mucous surface, chiefly due to gelatinous œdema with little leucocytic infiltration; soon, however, the mucous membrane becomes partially ulcerated, more or less extensive necrosis of the subjacent tissues occurs, and gangrenous sloughs result, the condition being aggravated by accompanying bacterial infection. The ulcers come to have irregular and overhanging margins, and the excavated membrane

but their most characteristic site is beyond the ulcerated area. may be seen penetrating deeply into the submucous and even into the muscular coats (Fig 191). In these positions they may be unattended by any other organisms, and the tissues around them show œdematous swelling and more or less necrotic change, without much accompanying cellular reaction beyond a certain amount of swelling and proliferation of the connective-tissue cells. These lesions are characteristic of amœbic dysentery. The amœbæ tend to penetrate into small vessels of the intestine (Fig. 191) and so get carried by the blood stream to produce metastatic lesions, e.g. in the liver. Infection of the skin may be caused by *E. histolytica*, e.g. around the anus or at the site of a cæcostomy.

Amœbic abscess. As a complication of this form of dysentery, liver abscess is of comparatively common occurrence. It is usually single and of moderate size. The contents are usually a

the only organisms present; occasionally secondary organisms occurs. The amœbæ are most numerous at the spreading margin, and this probably explains a fact pointed out by Manson, that examination of the contents first removed may give a negative result, while they may be detected in the discharge a day or two later. The action here on the tissues is of an analogous nature, namely, a necrosis with softening and partial

liquefaction, attended by little or no suppurative change. There is evidence, however, that the amœbæ may infect the liver without causing actual abscess formation, merely a hepatitis, and that this may be followed by cirrhosis. Abscesses are also met with in the lungs, as when a liver abscess has ruptured into the lung, which not very infrequently happens. There have also been recorded a considerable number of cases of cerebral abscess in which the amœbæ have been found, most of these have been secondary to lung infection.

Carriers. Cysts may be present in the feces of those who have never suffered from dysentery—'contact carriers', in them infection with the entamœba has occurred, and though slight lesions are probably present, they are not sufficient to give rise to symptoms. The occurrence of liver abscess has been recorded in such carriers. Wenyon and O'Connor examined the stools of nearly two thousand healthy British soldiers in Egypt and found that there was infection by *E. histolytica* in fully 5 per cent., while in less than a sixth of the infected individuals was there a history that they had suffered from dysentery. Among Egyptian natives 13.5 per cent were found to be carriers of *E. histolytica* cysts. Observations have shown that *E. histolytica* has a much wider distribution than was formerly supposed. Yorke, Mahins Smith, *et al.*, for example, have stated that the cysts are not infrequently to be found in various classes of the population in this country, the largest percentage of positives being among asylum inmates. According to the views of Brumpt the high proportion of European populations showing these cysts in the absence of cases of amœbic dysentery is due to their being infected with the non-pathogenic *E. dispar*, which cannot be differentiated morphologically from the smaller forms of *E. histolytica* and which also produces quadrinucleate cysts. It is stated to be incapable of producing severe lesions in kittens. At present it is impossible to decide whether it is a separate species or merely a permanently attenuated form of *E. histolytica*.



FIG. 191. Section of large intestine from a case of acute amœbic dysentery. Two specimens of *E. histolytica* are seen within a blood vessel in the muscular coat beneath the floor of an ulcer. Stained with iron haematoxylin. $\times 600$.

The extensive investigations of Dobell have shown that several species of *Macacus* monkeys in an apparently healthy state harbour naturally in their intestine amœbæ which in all their microscopic and cultural characters are identical with *E. histolytica*. Kittens were infected either by feeding with cysts contained in the monkeys' feces or by intrarectal inoculation with cultures from the litter; they acquired a subacute or chronic form of the disease, which points to the virulence of the monkeys' amœbæ being relatively low.

Experimental Inoculation. Dysentery develops occasionally in animals, e.g. in monkeys, but it is of comparatively rare occurrence. The disease may sometimes be reproduced in the dog by experimental inoculation with dysenteric material, as was shown first by Losch and afterwards by Kartulis, the latter producing the disease in one instance with the contents of a tropical abscess. Cats are, however, found to be more susceptible, especially young animals, and have been mainly used in investigation. Dysentery follows readily the introduction into the rectum of mucus from a human case, especially when the bowel is plugged, and a similar result has been obtained

by means of material from a living animal (quale). In the present case, since the amœbæ are destroyed in the stomach, as was shown by Quincke and Roos. The disease in cats experimentally produced by virulent amœbæ or their cysts is of an acute character and is usually fatal; in conformity with this, the cystic stage of the organism is not met with in the stools (*vide supra*).

The most important experiments were those carried out by Walker and Sellards on the human subject. They administered to Filipinos, who acted as volunteers, various amœbæ and entamœbæ or their cysts, the material being mixed with magnesium oxide or starch, and enclosed in gelatin capsules. Of twenty volunteers fed with motile *E. histolytica* or cysts, seventeen became parasitised after one feeding, the amœbæ appearing in the faeces after one to forty-four days (the average being nine days); of the remaining three, one was fed repeatedly and became infected after the third occasion. The amœbæ or cysts persisted for an indefinite period in the stools of those who became infected, four of them contracted dysentery, the average period of incubation being sixty-five days. In the case of the other amœbæ cultivable at that time, they found that though the organisms might be detected in the faeces after feeding with them, none of them became parasites and no pathogenic effects were produced. These results are of great importance both in demonstrating the specific pathogenic properties of the *E. histolytica*, and also in showing that it may become an intestinal parasite without causing dysenteric symptoms or gross lesions. Walker and Sellards concluded that the *E. histolytica* is a strict parasite, and that the source of infection is always another individual harbouring the organism in the intestine, and this view has received general support. An extensive epidemic of amœbic dysentery occurred in the United States in 1933 which was attributed to the water supply of two hotels in Chicago being infected from communication with the sewers. The original source of infection was not discovered, however. The incubation period in this epidemic was usually about two weeks, but in some cases dysentery developed within a week and in others not till after three or four months.

Macacus monkeys which had been freed from their natural infection by emetine were successfully reinfected by feeding with cyst-containing cultures of *E. histolytica* derived from other species; these cultures had been growing *in vitro* for up to one and a half years (Dobell). Amœbæ appeared in the faeces within four days. The infections thus produced were permanent, but no lesions developed. Similarly a culture derived from a man with acute amœbic dysentery proved infective for monkeys five years after isolation. As judged by the effects on kittens inoculated *per anum* with cultures of different strains of *E. histolytica*, there is no constant relation between cultivability, infectivity, and pathogenicity (Faust *et al.*, 1916). The influence which may be exerted by external factors, such as the associated bacteria, is still obscure.

Laboratory Diagnosis. The faeces in a suspected case of acute amœbic dysentery ought to be examined microscopically as soon as possible after defaecation.

In addition of any reagent, a cover-glass is placed over it, but not pressed down, and the preparation is examined in the ordinary way or on a warm stage, preferably by the latter method, as the movements of the entamœbæ become more active. Any specks of blood-stained mucus on the surface of formed stools should be examined. The characters of the nuclei are brought out by

emulsifying the specimen in Lugol's solution to examining for amœbæ, the c noted. The presence of abundant infection with *E. histolytica*, which absence of polymorphs indicates that the condition is not due to the dysentery bacilli. In the case of cysts, immediate examination of the specimen is not essential, as these persist unchanged for several days. The recognition of amœbic cysts and flagellates is aided by making a fine suspension of the specimen in a drop of 1 per cent watery solution of eosin on a microscope slide. This is best done by rubbing into the drop a small amount of the faeces on the cover-slip, care being taken that the suspension contains no other cells.

objective 1/8 inch 1/2 inch 1/4 inch 1/16 inch 1/32 inch 1/64 inch 1/128 inch 1/256 inch 1/512 inch 1/1024 inch 1/2048 inch 1/4096 inch 1/8192 inch 1/16384 inch 1/32768 inch 1/65536 inch 1/131072 inch 1/262144 inch 1/524288 inch 1/1048576 inch 1/2097152 inch 1/4194304 inch 1/8388608 inch 1/16777216 inch 1/33554432 inch 1/67108864 inch 1/134217728 inch 1/268435456 inch 1/536870912 inch 1/1073741824 inch 1/2147483648 inch 1/4294967296 inch 1/8589934592 inch 1/17179869184 inch 1/34359738368 inch 1/68719476736 inch 1/137438953472 inch 1/274877906944 inch 1/549755813888 inch 1/1099511627776 inch 1/2199023255552 inch 1/4398046511104 inch 1/8796093022208 inch 1/17592186044416 inch 1/35184372088832 inch 1/70368744177664 inch 1/140737488355328 inch 1/281474976710656 inch 1/562949953421312 inch 1/1125899906842624 inch 1/2251799813685248 inch 1/4503599627370496 inch 1/9007199254740992 inch 1/18014398509481984 inch 1/36028797018963968 inch 1/72057594037927936 inch 1/144115188075855872 inch 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in the fluid of the cultures the amoebae adhere to these and beautiful preparations may be obtained (Dobell).

NON-PATHOGENIC COMMENSAL AMOEBAE OF MAN

As has been mentioned above, other species of intestinal amoebae are met with in man. There are four—*Entamoeba coli*, *Endolimax nana*, *Iodamoeba*, and *Dientamoeba fragilis*. All of them are non-pathogenic commensals. In distinguishing them, the characters of the nuclei and cysts are of most importance. *Entamoeba gingivalis* occurs in the mouth.

Entamoeba coli. This is an intestinal commensal of common occurrence; it is of about the same size as *E. histolytica*, but on the whole is a little larger. When at rest it shows no differentiation into ectoplasm and endoplasm. The nucleus is readily seen, and shows a highly refractile membrane with chromatin lining it and in the interior; the karyosome, which is larger than that of *E. histolytica*, is placed excentrically in the nucleus. The characters of the nucleus, as seen in fixed and stained preparations, are shown in Fig. 189, G. The protoplasm has a granular appearance, and in it there are often small vacuoles containing bacteria, food particles, etc.; glycogen also is present. During amoeboid movement, which is usually sluggish, some delicate processes of ectoplasm come into view, but the characteristic movements described above in the case of the *E. histolytica* are not met with. It is generally stated that red corpuscles are not found in the interior, though bacteria, food particles, etc., are often abundant. Dobell has pointed out, however, that the cultivated amoebae may ingest red cells as readily as *E. histolytica*. The cellular changes in the encysting of the *E. coli* correspond with those of *E. histolytica*, and the ultimate result is the formation of a fairly large cyst (Fig. 190 V W) which contains from two to eight nuclei. As seen in the fresh state the cyst-interior

those of the vegetative form of the amoeba. Glycogen is abundant in the vegetative form, but absent in the cysts, but elongated or bar-shaped chromatoid bodies are usually absent. They are in these ways distinguishable from the cysts of *E. histolytica*.

In the case of the *E. coli*, Walker and Sellards were able to bring about parasitism by feeding with the cysts of the organism, but no pathogenic effects followed. Their results accordingly confirm the view previously held, that it is a harmless organism. It has practically a world-wide distribution, and in certain countries is very common. Schaudinn found that in East Prussia as many as 50 per cent. of the population were infected with it, and confirmatory results with regard to its common occurrence were obtained by Craig in San Francisco.

Endolimax nana (Entamoeba nana). This is a small amoeba about 6 to 15 μ in diameter, on the average 8 to 10 μ , and somewhat resembling a small *E. coli* in its appearance and movements. Like the latter, also, it often has in its protoplasm vacuoles containing ingested bacteria. In fixed and stained preparations its small nucleus is characterised by its relatively large karyosome, which varies in shape and in its position within the nucleus (Fig. 189, D, E). The amoebae are not infrequently parasitised by a small micro-organism belonging to genus *Sphaerita*, the small oval and highly refractile spores of which form regular cluster-like masses of eight (Dobell). The cysts of *E. nana* are of about the same size as the free forms, and when mature contain four minute nuclei, each having the peculiar character referred to (Fig. 190, S, T, U); they contain small granules or rods. *E. nana* is a common commensal of the human intestine, and has a wide geographical distribution; it has often been found in the faeces of animals who have never been abroad. Though it has been met with most frequently in conditions of intestinal disorder, it also occurs in quite healthy

individuals, and there is no evidence that it has any pathogenic properties (For the cytology and life history, see Dobell)

Iodamoeba bütschlii. As seen in the fresh faeces, the organisms measure 8 to 12 μ in diameter and resemble somewhat small specimens of the *E. coli*. As in the latter, there is little differentiation between ectoplasm and endoplasm, and within the endoplasm there are many vacuoles containing ingested bacteria, etc. The nucleus is vesicular and contains a large central karyosome (Fig 189, F). The cysts are of about the same size as the free forms. Each cyst contains a single nucleus in which a similar karyosome is situated at the periphery, and usually also a comparatively large mass of glycogen (Fig 190, G, R). This species is of less common occurrence than the two previously described.

Dientamoeba fragilis (Jepps and Dobell). This organism, which is rare, is distinguished by usually possessing two nuclei of similar structure, though uninucleate forms also are met with. It is of small size, measuring 5 to 11 μ in diameter. Each nucleus (1.5 to 2 μ in diameter) is vesicular and contains a central karyosome of granular structure with a clear zone around it (Fig 189, H, I). No cysts of this organism have been found and Dobell has concluded from its cytology and development that it is an aberrant flagellate.

Entamoeba gingivalis. The occurrence of amœbæ in the mouth has long been recognised, but special attention has been directed to them in view of their occurrence in pyorrhœa. The amœbæ, so far as is known, all belong to the one species—*E. gingivalis* (also called *E. buccalis*). The organism is a small amœba which tends to abound in certain morbid conditions of the mouth. It is of smaller size than the *E. coli*, measuring usually about 10 to 20 μ in diameter. The nucleus, seen with some difficulty in the living amœba, is like that of *E. histolytica*, it is relatively small, measuring 3 to 4 μ . The amœbæ show active amœboid movements, throwing out rounded pseudopodia, and ectoplasm and endoplasm are clearly distinguishable. The endoplasm is granular, and often contains numerous rounded bodies or inclusions, which stain deeply with nuclear dyes, and probably represent the remains of nuclei of leucocytes or other cells. No cysts of this organism have been observed. Although the amœbæ have been found in a fair proportion of healthy individuals, there is no doubt that they are specially abundant in morbid states. They are usually present in pyorrhœa, and are commonly met with in dental caries, and in the tartar of the teeth. It has not been established that the organism is responsible for pyorrhœa or any other morbid change, and the evidence goes to show that it is a fairly common commensal of the mouth, which becomes more numerous in pathological conditions.

FLAGELLATES OF THE HUMAN ALIMENTARY AND GENITO-URINARY TRACTS

These motile organisms are adapted for life in a fluid medium, which may explain the vegetative forms being found in the intestinal contents in conditions of diarrhœa more readily than normally. According to some they may cause inflammation of the mucous membrane, but it is not proved that they invade the bowel wall. They are examined by the same methods as the entamoebæ. They have all been cultivated except *Giardia*.

Giardia intestinalis (Lamblia) (Fig 192, 1) is bilaterally symmetrical and 10 to 20 μ long. It is thick and rounded anteriorly and tapered posteriorly. The ventral surface is hollowed anteriorly to form a 'sucking disc', which is surrounded by deeply stained fibres. There are two nuclei, and four pairs of flagella. In formed faeces only the cysts are found (Fig 192, 2-3). The vegetative form lives in the small intestine especially the duodenum.

Trichomonas hominis (Fig 192, 4-5) is oval 7 to 15 μ long. A characteristic structure is the undulating membrane, which runs lengthwise along the surface. The nucleus is placed anteriorly, where there are also 3 to 5 free flagella arising from a blepharoplast, while another passes back in a wavy line, attached along the undulating membrane and becoming free at the posterior end of the organism. There is a cleft-like 'mouth' situated alongside the nucleus. A rod-like structure the axostyle, runs longitudinally down the middle of the body and ends as a spike like projection. Degenerate forms may be recognised by a rippling movement which occurs along one side. The cysts are not known. *Trichomonas buccalis* (*T. tenax*) is found in the mouth and *Trichomonas vaginalis* in the vagina and also, in both sexes, in the urethra and adnexa. These are believed by some to be distinct species on morphological and other grounds. On the other hand, Dobell with *T. hominis* cultivated from human faeces produced a persisting infection in the vagina of a *Macacus rhesus* monkey. There is clinical evidence that *T. vaginalis* acts as a pathogen in the vagina and urinary tract. For the detection of *T. vaginalis* a wet film should be made and examined at once or a thin film of the discharge

(diluted if necessary with 10 per cent. human serum in saline) which has been rapidly dried, is stained with Leishman's solution; cultivation may also be employed (see Linton).

Chilomastix mesnili (Fig. 102, 10) is similar to *T. hominis* in size, but is more rigid owing

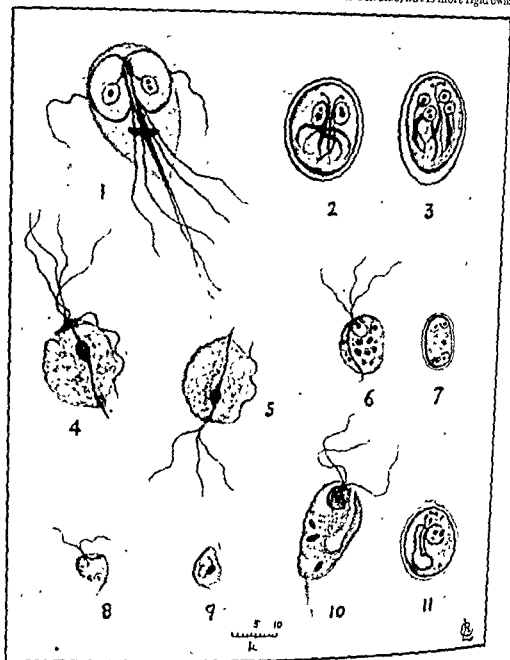


FIG. 102 Intestinal flagellates of man

flagella.

From fixed specimens stained with iron haematoxylin $\times 1,000$

to a surface pellicle, it has a caudal spike. The 'mouth' is large and conspicuous. The cysts are shaped like a lemon or grape seed and have a single nucleus, the karyosome showing often the 'signet ring' disposition (Fig 102, 11). This is a common intestinal parasite.

Embadoromonas intestinalis (Retortamonas) (Fig 102, 8) is small, 4 to 9 μ in its longer axis and has two flagella placed anteriorly. The cysts are like those of *C. mesnili*, but smaller, 4.5 to 6 μ (Fig 102, 9). This parasite is uncommon.

Enteromonas hominis (*Tricercomonas intestinalis*) (fig. 192, 6) is oval, 4 to 10 μ long with three free flagella anteriorly, where the nucleus is also placed and a fourth which passes back along the body to become free for a short distance. There is no mouth or undulating membrane. The cysts are oval, 6 to 8 μ long, and when mature have four small nuclei with a central karyosome (fig. 192, 7).

BALANTIDIUM COLI

This protozoan belongs to the class of the Ciliata. It occurs commonly in the intestine of pigs as a commensal and is also found in other animals. In man infection which is not very frequent, results from swallowing cysts. This tends to cause ulcers similar to those of amoebic dysentery, the balantidia being found in the depths of the

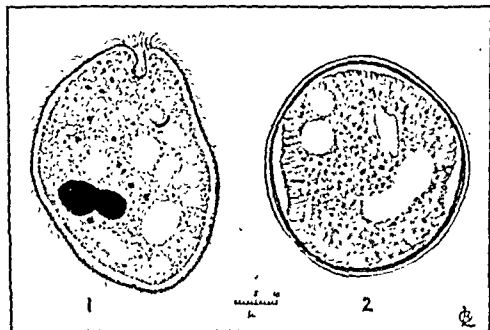


FIG. 193. *Balantidium coli*. 1 From human feces, fresh and stained. $\times 1,000$. 2 Large cyst from pig feces. $\times 1,000$.

lesions. The vegetative form of the organism (fig. 193, 1) is found in the stools when there are dysenteric symptoms. In the fresh state it appears greenish. It is oval measuring on the average 60 to 70 μ by 40 to 50 μ , but larger and smaller forms occur. There is a slit-like mouth near the more pointed end. The body is somewhat rigid owing to a pellicle through which project parallel rows of aniferous cilia distributed all over the surface. These cause it to be actively motile. There are two nuclei, one being the kidney-shaped macronucleus in the cavity of which a micronucleus lies. There are also two contractile vacuoles, as well as food vacuoles, and red blood corpuscles may be ingested. The cysts (fig. 193, 2) which measure 50 to 60 μ are greenish yellow and have a double wall through which the structure of the organism can be seen. Cultures have been obtained. In cases with typhoid stools the cysts alone are found. In examining feces for *Balantidium coli* care must be taken that the specimen be mixed with water, which often contains some other singular protozoa.

CHAPTER XXXV

PATHOGENIC PROTOZOA (*contd.*)—TRYPANOSOMES (*T. GAMBIENSE*: *T. RHODESIENSE*: *T. CRUZI*: ANIMAL TRYPANOSOMES)

THE trypanosomes are protozoal organisms belonging to the class Flagellata (Mastigophora). Members of the genus *Trypanosoma* live in the blood and tissues in various animals and certain of them cause important disease conditions. These diseases have general resemblances to one another. They tend to be characterised by wasting, cachexia, anæmia, fever, often of an intermittent type, and irregular œdemas, and frequently have a fatal result—the condition in domestic animals being called nagana. The infective agent in trypanosomiasis of mammals has been proved frequently to be conveyed from a diseased to a healthy animal by the agency of blood-sucking insects (especially tsetse flies), the protozoon in some cases undergoing a cycle of development in the latter. Under experimental conditions infection can be transmitted from one animal to another.

In 1

ense, *T. rhodesiense*, and *T. cruzi*: the first two, the most important causes of trypanosomiasis, occur in Africa, producing trypanosome-fever and sleeping sickness.

MORPHOLOGY AND BIOLOGY. If a drop of fresh blood containing trypanosomes be examined between a slide and coverslip, the organisms are seen to be fusiform, one end passing into a pointed flagellum. In the living condition the trypanosomes are usually actively motile by an undulatory movement of their protoplasm and a lashing of the flagellum, but often there is little tendency to movements of progression. From the fact that in progression the flagellum is in front, the end at which it emerges is regarded as anterior. The size varies, but many, including those parasitic in man, are about 12 to 35 μ long and about 1.5 to 3 μ broad. Examination of fresh blood is the one most likely to reveal the presence of trypanosomes, if these are present in small numbers, since they are readily detected by the movement which they impart to the adjacent red corpuscles. But the minute structure of the organisms can be studied best in preparations fixed after drying or preferably when wet, and stained by Romanowsky dyes, such as those of Leishman or Giemsa. When stained by the above methods the protoplasm of trypanosomes appears blue, and in certain species some parts are more intensely coloured than others. Sometimes it contains violet-coloured granules (volutin granules), and occasionally there appears in it slight longitudinal striation. Two bodies are always present in the protoplasm. An oval kinetoplast, is usually near the anterior end, and is intensely stained.

purple granule known as the kinetoplast, which may be differentiated into the blepharoplast (the granule from which the axoneme arises—*vide infra*), and deep staining material, the parabasal body. The micronucleus is often surrounded by an unstained halo, and in its neighbourhood in certain species a vacuole has been described. From the micronucleus or from its neighbourhood there arises an important structure in the trypanosome—the undulating membrane. This is of varying breadth, has a sharp, undulating, free margin, and surmounts the protoplasm of the

organism like a cock's comb, it narrows towards the anterior end, where it becomes the flagellum. A filament (axoneme) which stains of the red tint of chromatin, commences at the blepharoplast and runs along the free margin of the undulating membrane, it then forms the core of the flagellum. The latter is continuous with the protoplasm of the body of the organism. Motion is chiefly effected by the undulations of the membrane and of the flagellum. Different species of trypanosomes exhibit variations in shape, in length, in breadth, in the position of the micronucleus (and therefore in the length of the undulating membrane), in the breadth of the membrane, in the length of the free part of the flagellum, in the shape of the posterior end, which is sometimes blunt, sometimes sharp, and in the presence or absence of free chromatin granules in the protoplasm.

Multiplication in the body fluids ordinarily occurs by longitudinal, amitotic division, the parasite having increased in length and breadth beforehand. First of all, the micronucleus divides, sometimes transversely, sometimes longitudinally, then the macronucleus and undulating membrane, and lastly the protoplasm. In some species only the root of the flagellum divides, so that in the young trypanosomes the flagellum is short and subsequently increases in length (*T. lewisi*). It has been held that the whole flagellum takes part in the general splitting of the organism, but according to Wenyon, after the blepharoplast has divided, the original axoneme remains attached to one moiety and a new axoneme grows out of the other. The view has been advanced that in certain cases reproduction occurs by the formation of 'latent bodies' consisting of the nucleus fused with the blepharoplast (Moore and Breinl), or by the endogenous formation in the nucleus of 'chromidial buds' (Muchan) or 'infective granules' (Henry and other observers), which when extruded from the protoplasm develop into trypanosomes, but according to other workers these appearances may be due to degenerative changes.

The morphology of certain species of trypanosomes tends to vary, and this feature is more or less marked according to the host which they are infecting. In man both *T. gambiense* and *T. rhodesiense* are practically monomorphic, but when transferred to laboratory animals they become polymorphic and in the circulating blood show differences in shape and size. There is a form long and slender in both body and nucleus, the free part of the flagellum being longer than the body and the protoplasm devoid of granules. At the same time a broader form of the organism with a larger and rounder nucleus and a blunter posterior extremity is found; its undulating membrane is narrow and the free part of the flagellum is shorter than the body, and the protoplasm contains granules. According to one view, this polymorphism indicates sexual differentiation, the former being the male form and the latter the female, but intermediate forms also occur, and conjugation has never been observed. Accordingly, it is probable that the variations indicate merely different stages of growth. Whether any pathogenetic significance is to be attached to the occurrence of these different forms is at present unknown. In the case of *T. rhodesiense* especially, posterior nuclear forms are met with in which the micronucleus is situated toward the posterior end of the parasite close to the flagellum.

EXCITIVITY AND PATHOGENICITY TO DIFFERENT VERTEBRATES. Some trypanosomes, such as *T. lewisi* and *T. tritrichi*, infect only vertebrate species, others a very broad range of other animal groups. *T. tritrichi* is especially pathogenic to the lower forms of species of *Amphibia* and *Reptalia*, such as *T. lewisi*, *T. gambiense* and *T. rhodesiense* infect only the higher forms of *Reptalia* and *Aves*, and also, as noted, of *Mammalia*.

containing the parasites. While some species of trypanosomes give rise to serious disease, in the case of others a heavy infection may occur without the animal suffering any apparent inconvenience; again, a form producing disease in one species of host, may be present in considerable numbers in another species without causing any pathogenic effects. Certain trypanosomes, e.g. *T. brucei*, when passed repeatedly through rats or mice, by injecting parasite-containing blood, become highly virulent for the specific host and increase progressively in the blood, thus producing a rapidly fatal general infection with a 'septicæmic' course. On the other hand, in rabbits a chronic relapsing infection is produced by several of the pathogenic trypanosomes, which is similar to the clinical disease in the larger domestic animals and man. The capacity to acquire virulence for a specific host is a property inherent in the trypanosome, thus *T. rhodesiense* is more virulent for laboratory rats and mice than *T. gambiense*, but inferior in this respect to *T. brucei* when fully accommodated and virulent.

In chronic infections the number of organisms present in the peripheral blood varies, and thus the potentiality of infection by means of an invertebrate carrier also varies. When the organisms are absent from the blood they may still be found in the solid organs and in the bone marrow, and in such situations may perhaps go through a phase of development. In *T. cruzi* such a stage has been clearly demonstrated in tissue cells.

Small animals with a septicæmic form of the infection tend to die suddenly with convulsions. The blood sugar in such animals is low, and as the parasites have an active sugar metabolism, it is possible that this accounts for the hypoglycæmia and contributes to the fatal result. The means by which trypanosomes produce their chronic pathogenic effects are unknown. Toxic substances are stated to have been obtained from the bodies of the parasites.

TRANSMISSION. The outstanding fact in the biology of the pathogenic trypanosomes is that infection from vertebrate to vertebrate usually takes place through the agency of biting or blood-sucking insects, or, in the case of frogs and fishes, by leeches. The mere mechanical transference by such invertebrates is possible, and in certain cases multiplication of the organisms in the biting apparatus of the invertebrate occurs. Such a mechanical or semi-mechanical transference, however, plays only a subsidiary part in spreading the infection in many cases, including the human trypanosomes, since a considerable period elapses before an insect which has ingested infected blood becomes infective for new hosts. Here the parasite undoubtedly goes through a cycle of development within the invertebrate, the details of which are in some instances as yet undetermined. In the alimentary tract of the insect, the trypanosomes are seen to undergo modifications in form. They may show simple division, by which the resulting individuals become smaller, the relation of kinetoculus and macronucleus may be altered, the former coming to lie anterior to the latter, while the undulating membrane and flagellum become rudimentary (crithidial forms). In other cases, organisms resembling leishmanæ result. The behaviour of the organisms in the invertebrate host before they again become infective for vertebrates varies in different instances. Thus *T. gambiense* passes finally to the salivary glands of the tsetse fly (the 'anterior station'); on the other hand, *T. lewisi* travels to the rectum of the rat flea (the 'posterior station') and the infective forms pass out in the faeces. The analogy of what happens in the malarial parasite suggests the possibility of a sexual element in a trypanosomal cycle, and Koch and Kleine found in the intestine of the insect vectors agglomerations of immature forms which they ascribed to the results of

sexual conjugation. But there is at present no definite proof that such a stage has ever been observed. The trypanosomes are not transmitted hereditarily by infected insects. Both sexes act as the vectors.

IMMUNITY. TRYPANOCIDAL ACTION OF SERUM. In the case of small animals in which the infection runs an acute septicæmic course, e.g. *T. brucei* in the mouse, immunity phenomena can be demonstrated after cure by a chemotherapeutic agent. On reinoculation with the homologous strain infection does not develop, or does so only after a prolonged incubation period. Again, when an animal is inoculated with a species of trypanosome to which it is moderately susceptible, e.g. *T. brucei* in the rabbit, it usually happens that after trypanosomes have appeared in the blood and become numerous they diminish more or less critically, thereafter the number of parasites fluctuates and the infection pursues a subacute or chronic course. The most advantageous outcome for the host under such circumstances is that, short of ultimate sterilisation, equilibrium with the parasites is established, so that no pathogenic effects occur, this is referred to as latent infection or premunition. There is at this phase a relative immunity to reinoculation with the same strain. Cure by a drug in this case is often followed by prolonged resistance to reinoculation with the homologous strain. The serum of recovered animals contains antibodies which may produce various effects on the trypanosomes. *In vitro* there are observed, agglutination, lysis, or trypanocidal action, also, under the influence of the specific antibodies, e.g. when mixed with citrated blood of an immune animal, the homologous trypanosomes in a drop of blood of an infected animal quickly become covered with blood platelets (the adhesion phenomenon of Rieckenberg)—other small particles such as certain bacteria or human red cells adhere similarly when incubated along with the trypanosomes and the homologous antiserum in the presence of complement (see Brown and Broom). *In vivo* the antiserum confers on a susceptible individual resistance to infection or has a therapeutic effect in established infections.

When a relapse occurs, either after administration of a non curative dose of a chemotherapeutic agent, which has caused the trypanosomes to disappear temporarily, or when the same result occurs spontaneously, the parasites which reappear in the blood are serologically different from those previously present. Inoculation of such a 'relapse strain' into an animal cured of the original infection leads promptly to appearance of trypanosomes in the blood, as in a normal susceptible animal. Thus the relapse strain is immunologically distinct from the original, being resistant to the antibodies developed in response to the original infection. A point of great importance, also, as bearing on the occurrence of relapses, is that brief contact *in vitro* of the antiserum with the parasites renders the latter serum resistant (Ehrlich, Roehl, and Gulbransen). On account of this tendency of trypanosomes to undergo changes in their antigenic constituents and so to alter in their behaviour to antisera, serological methods cannot be relied upon for distinguishing different species of those organisms.

In connection with natural immunity to trypanosomes it is of interest that normal human serum has a marked trypanocidal action on various trypanosomes pathogenic to animals, e.g. *T. brucei*, *T. congolense*, and *T. equiperdum*, but it is without action on *T. gambiense*, *T. rhodesiense* when first transmitted from man to experimental animals may be unsusceptible to human serum, but soon becomes susceptible. The mechanism of this trypanocidal action of human serum appears to be different from that of bactericidal action. Differences in susceptibility to human serum, however, do not wholly account for the differences in infectivity of a strain of *T.*

brucei resistant to human serum, but on inoculation it proved to be non-infective for human volunteers. Further, although baboons are resistant to *T. gambiense* inoculated into the blood stream, their serum is only weakly trypanocidal. It is of interest in connection with their natural immunity that Regendanz succeeded in producing a fatal infection of the central nervous system in baboons by intraspinal inoculation with *T. gambiense*.

IDENTIFICATION OF TRYPANOSOME SPECIES. The differentiation of species of trypanosomes may present great difficulties. Both morphological and biological characters must be taken into account. As has been seen, *T. gambiense* and *T. rhodesiense* are similar in their appearance in the blood in man, but differ in experimental animals, in which *T. rhodesiense* alone shows frequent posterior nuclear forms. Again, *T. gambiense* differs from all other morphologically similar trypanosomes in being insusceptible to the trypanocidal action of human serum. Biological characters which are important for identification are the species of susceptible vertebrates and the course of the disease in these; the species of the insect vectors; the behaviour of the parasites both in vertebrate and invertebrate hosts, and the response of infected animals to administration of trypanocidal drugs. Immunity tests may not be of value, since trypanosomes are capable of great antigenic variation, and parasites derived from a relapse are frequently serologically different from those of the strain causing the original infection (cf. relapsing fever spirochaetes, p. 195). On the basis of such cumulative evidence it is held that, for example, *T. gambiense*, *T. rhodesiense*, and *T. brucei* represent biological races of a single species (Hoare).

CULTIVATION. A number of the trypanosomes have been cultivated outside the bodies of their natural hosts, the first work having been done with the rat trypanosome, *T. lewisi*, by Novy and MacNeal, who introduced a special medium for the purpose.

Nicoll's modification of Novy and MacNeal's blood agar ('N N N medium') for the cultivation of trypanosomes, leishmaniae, etc., is prepared as follows. Mix thoroughly in a flask 14 grms agar, 6 grms NaCl, and 900 c.c. water, and steam for two hours (the medium is not neutralised or cleared). Filter through cotton wool and tube 2 c.c. in tubes of $\frac{1}{2}$ -inch diameter, autoclave at 120 C for twenty minutes. Cool the medium to 50 C in a water bath, then into each tube introduce 20 drops of whole rabbit's blood obtained with aseptic precautions (vide Appendix). Mix thoroughly by 'rolling' in the hand, and allow the medium to solidify in the sloped position. Incubate the tubes for several days at 37 C to test sterility. As the presence of abundance of condensation water is essential, evaporation must be prevented by capping the tubes. Inoculation is made by introducing the infective material by means of a capillary pipette into the water of condensation.

Brutsaert and Henrard have obtained cultures of *T. gambiense* by the following method (see Hawking). 0.2 c.c. of infected blood (rendered incoagulable by the addition of 1 c.c. 1 per cent 'liquid' (Roche) solution to 5 c.c. blood) is added to a mixture of 1 c.c. 1 per cent sodium citrate solution and 1 c.c. 1 per cent sodium citrate solution. Incubate for twenty days.

In cultures, the organisms may divide longitudinally, as seen in the blood, or crithidial or leishmania form, may result, the former being often arranged in rosettes containing a large number of individuals with their flagella orientated in the same direction. A fresh infection may sometimes be originated by introducing cultures into suitable animals. But in the case of the human trypanosomes cultures are difficult to establish and usually are not infective.

Multiplication of various trypanosomes has been obtained in the allantoic cavity or yolk sac of fertile eggs.

TRYPANOSOMA GAMBIENSE

Since the year 1800 the disease called sleeping sickness, sleeping dropsy, or negro lethargy has been recognised as prevailing on the West Coast of Africa from the Senegal to Lagos, and in the parts lying behind the coast between these regions. It has also been found to be rife from Cameroon to Angola, and in the Congo valley, and to a less extent up the Niger and its tributaries. In 1901 it began to appear in the Uganda Protectorate, where it has wrought very serious havoc among the native population, and the investigations carried on in that region have led to a knowledge of its cause. A local inflammatory reaction of the skin occurs at the site of the infective bite, this disappears in several weeks. After an incubation period of about a fortnight following inoculation by the bite of the fly, fever develops, the evening temperature may be elevated several degrees. There may also be headache, hyperæsthesia, and indefinite pains about the body, the pulse tends to be soft and rapid, and in a very large number of cases the superficial lymphatic glands are enlarged. The initial features preceding the onset of the sleeping sickness stage are a change in disposition leading to moroseness, apathy, disinclination for work or exertion, and slowness of speech and gait. In a rapid case lethargy soon develops and deepens, fine tremors, especially of the tongue and arms, appear, progressive emaciation occurs. Blood changes arise, consisting of a progressive diminution of the red cells and of the hæmoglobin, and of a lymphocytosis in which the percentage of both the large and small mononuclear cells is increased, so that the former may constitute from 20 to 30 and the latter from 30 to 40 per cent of all the white cells present. As the illness progresses the drowsiness increases till the individual lapses into a coma from which he cannot be roused. Often there occur irregular cedematous patches on the skin, and sometimes erythematous eruptions, and effusions into the serous cavities. The disease has a high fatality, and probably where the actual lethargy is developed recovery never occurs. Not every case runs a progressively advancing course such as that described above. Sometimes the chief early feature, besides enlargement of glands, is the occurrence from time to time of attacks of fever which may be mistaken for malaria, and from these apparently complete recovery may take place, recurrence, however, follows as a rule, and ultimately the typical terminal phenomena commence. Such cases may go on for years and it is likely that many patients die of pneumonia or some other infection without exhibiting typical manifestations of the malady from which they really suffer.

Owing to the seriousness of the epidemic of sleeping sickness in Uganda, a Commission of the Royal Society was dispatched in 1902 to investigate the condition. Castellani found in some cases in the cerebro-spinal fluid, especially when this was centrifuged, living trypanosomes resembling the *T. gambiense* which seem at first to have been regarded as accidental. Bruce pursuing the work of the Commission with Nabarro and Greig in 1903 made a series of examinations in several infected localities and demonstrated the trypanosome in every case of the disease. This work formed the starting point for inquiries which proved that the parasite is the causal agent of the condition. The organisms were not seen in the cerebro-spinal fluid of patients dying of other diseases in the sleeping sickness area. On the other hand it was found that if cerebro-spinal fluid withdrawn from cases of the disease was injected into monkeys (especially *Macacus rhesus*) trypanosomes appeared in the blood and in many cases in three or four months the animals died of an illness indistinguishable from sleeping sickness, and with the

parasites in the central nervous system. On inoculation of other species of animals, *e.g.* herbivora or the guinea-pig, in nearly every case a proliferation of the parasite takes place, as indicated by its appearing in the blood, but often either no disease occurs or this runs a very chronic course. Small animals such as rats and mice can also be infected, *e.g.* by subcutaneous inoculation. Under ordinary conditions the trypanosomes do not long survive removal from the body.

Pathological Changes. As described by Mott, the most striking pathological change in cases of sleeping sickness is the presence of a chronic meningo-encephalitis and meningo-myelitis. The pia-arachnoid is sometimes opaque and slightly thickened and may be adherent to the brain, and its vessels usually show some congestion. The sub-arachnoid fluid is sometimes in excess and occasionally may even be turbid. The membranes of the spinal cord show similar changes. The chief other feature is the presence of enlarged lymphatic glands in the body, but otherwise there is nothing special to note. With regard to the microscopic changes, the chief feature is a proliferation and overgrowth of the neuroglia cells, especially of those which are related to the sub-arachnoid space and also the perivascular spaces, with accumulation and probably proliferation of lymphocytes in the meshwork. The changes in the lymph glands are of similar nature, and resemble the infiltration of the perivascular spaces of the central nervous system. These changes are specially significant in view of the lymphocytosis present in the blood, which has already been noted, and which so often occurs in protozoal infections. In the nerve elements there are merely some atrophy of the dendrons of the nerve cells, a diminution of Nissl's granules, and an eccentric position of the nuclei.

Trypanosome Fever The first case in which trypanosomes were found in the blood was recorded by Forde and Dutton in 1902, the patient, who was not obviously suffering from

Bathurst on the Gambia. The case was characterised by general wasting and weakness, irregular rises of temperature, local oedemas, congested areas of the skin, enlargement of spleen, and increased frequency of pulse and respiration. Death occurred a year after the patient came under observation, following an access of fever, and a striking fact was the absence of any gross lesion. During the period of observation trypanosomes were repeatedly demonstrated in the peripheral blood, and they also developed in monkeys and white rats inoculated with the blood. Pursuing further inquiries, Dutton and Todd demonstrated similar parasites in other Europeans and in several natives in the Gambia region, while about the same time Manson reported a case of the same kind from the Congo. It thus came to be recognised that in man there occurred a disease somewhat resembling nagana, in which trypanosomes were present in the blood, and this was usually referred to as human trypanosomiasis, or trypanosome fever -- the trypanosome being named the *T. gambiense*.

Early in the Uganda investigations the question arose as to whether the trypanosome of sleeping sickness was different from that discovered by Dutton. This was forced on the inquirers by the fact that a large proportion of the natives in the sleeping sickness area were found to harbour trypanosomes in their blood, although not apparently suffering from the disease. Several cases were carefully examined in which trypanosomes were constantly present in the blood, but in which the patients from time to time suffered from fever, and during these pyrexial periods trypanosomes were found in the cerebro-spinal fluid. It was suggested that these cases were on the way to develop sleeping sickness. A very important observation was that while in

sleeping sickness areas a large proportion of the native population harboured trypanosomes, this was not the case where sleeping sickness did not occur. Further, it was found that trypanosomes from the cerebro-spinal fluid of sleeping sickness cases and from the blood of persons harbouring trypanosomes, but not suffering from disease symptoms, gave rise in monkeys to the same group of chronic effects which resembled the last stages of the disease in man. These facts led the Commissioners to incline to the view that trypanosome fever and sleeping sickness are due to the same cause, and represent different stages of the same disease. It has already been pointed out that a fatal termination can occur in trypanosome fever by an acute febrile attack or from intercurrent disease, and thus the terminal lethargic stage may only develop in a certain proportion of cases. Continued observation of prolonged cases of trypanosome fever, both in Uganda by Greig and Gray, and in this country by Manson, has shown that sometimes the termination of a case is by the onset of typical sleeping sickness. There is now practically no doubt that the two conditions are etiologically identical. It is generally agreed, also, that there are no morphological differences between the trypanosomes from the two types of case, hence the name *T. ugandense* originally applied to the organism recovered from actual cases of sleeping sickness is no longer used.

The prevalence of trypanosomes in the blood of apparently healthy natives has raised the question of the possibility of tolerance existing and of immunity being established. It is possible that both phenomena occur, that not every infection results in multiplication of the parasite in the body of the victim, and that in certain cases where multiplication does occur, a resistance is developed which enables the body to kill the parasites. It has been suggested that when this resistance is weak the organism gains entrance to the cerebro-spinal system, and that then sleeping sickness results.



FIG. 191. *Trypanosoma gambiense* from blood of human case. Leishman's stain. $\times 1,000$.

MICROSCOPIC CHARACTERS. Since *T. gambiense* usually occurs only in small numbers in the blood in man (Fig. 191), its microscopic characters have been studied mainly in infections of animals such as guinea-pigs or rats. In these it measures on the average 15 to 30 μ , the nucleus is central and the blepharoplast close to the posterior end. The trypanosome is polymorphic, long slender forms with flagella and short broad forms without free flagella, as well as forms of intermediate length, being found (Fig. 193, 1-5). In man the parasites also penetrate into the tissues, especially muscle.

Transmission by Tsetse Flies (Glossinidae). It was found that in the parts round the north end of Lake Victoria Nyanza where sleeping sickness was prevalent, the distribution of the disease exactly corresponded with the distribution of a blood-sucking insect, the *Glossina palpalis* (a species closely allied to the *Glossina morsitans* of nagana). When one of these flies was fed on a sleeping sickness patient and then allowed to bite a monkey, trypanosomes frequently appeared in the animal's blood, and the same result often followed when fresh flies caught in the sleeping sickness area were placed on a monkey. Kleine established the important fact that when *G. morsitans* was allowed to bite an animal suffering from nagana the fly did not become infective for about twenty days. This was confirmed for *G. palpalis*,

in the case of monkeys infected with *T. gambiense*, by Bruce and those associated with him. Here it was found that infectivity did not appear till about thirty-two days after the fly had fed, and continued until at least seventy-five days. It was at first supposed that monkeys could not be infected with the trypanosomes from the bruised-up bodies of the fly, but Bruce succeeded in originating an infection with this material, results being positive during the first two days after the fly had bitten and then being negative till after the twenty-second day. Bruce noted that the renewed infectivity corresponded with the appearance of 'metacyclic' trypanosomes in the salivary gland of the glossina. The cycle of development of the trypanosome in *Gl. palpalis* has been found by Bruce and his co-workers and by Robertson to be as follows. Thirty-six to forty-eight hours after ingestion of the infected blood, many of the parasites are degenerate, but some of the broad forms are dividing; the products of division show for a short time crithidial forms in which the blepharoplast is situated anteriorly and the undulating membrane is only slightly developed. At the tenth day numerous trypanosomes of very variable shape and size are present; later, slender forms appear in increasing numbers, and these pass to the proventriculus and hypopharynx. They travel along the duct to the salivary gland, and here crithidial forms develop, the latter again produce trypanosomes, and after the appearance of these 'metacyclic' forms at about the twentieth day the fly becomes infective for the vertebrate host. Only a small proportion of flies which have ingested trypanosomes in the blood of a patient become infective subsequently. The temperature has an important influence on the development of the trypanosomes in the fly; thus Kinghorn and Yorke found in the case of *T. rhodesiense* that under 21° C. the trypanosomes did not invade the salivary glands of *Glossina morsitans*, and hence the fly did not become infective.

Certain facts having an important bearing on the continued infectivity of a locality have emerged. It was found that a certain island on Lake Victoria Nyanza, which had been cleared of infected natives two years previously still harboured infective flies. To account for this it must be supposed either that the *Glossina* has an extended duration of life, or that the trypanosome exists among the wild animals. Cattle and wild herbivora, especially certain antelopes, can be infected with the parasite, and can through the medium of the fly infect monkeys. It is possible that such animals, while not suffering in any serious way themselves, are the means of maintaining infectivity. In certain areas, apparently healthy pigs have been found to act as a reservoir of *T. gambiense* from which flies were readily infected (van Hoof *et al*). There is no definite evidence that, as Koch supposed, the crocodile harbours the trypanosome.

In some districts the infection may be transmitted by *Glossina tachinoides* and possibly by other species of *Glossina*.

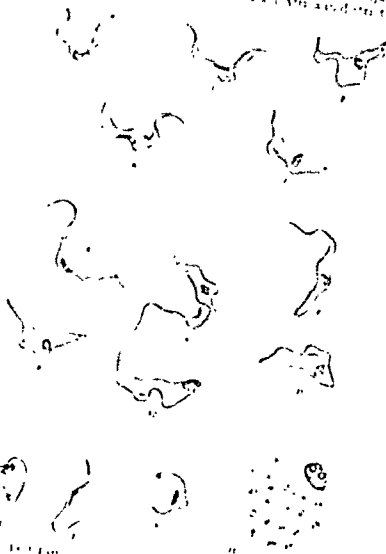
TRYPANOSOMA RHODESIENSE

In 1910 Stephens and Fantham observed certain peculiarities in the trypanosomes derived from a case of human trypanosomiasis occurring in an individual who had returned to England from Rhodesia. The organisms in the blood of small animals (rats, mice, and guinea-pigs) frequently presented a very blunt posterior end and the macronucleus tended to approach the kinetonucleus and might lie behind it—posterior nuclear forms (Fig. 195, 6-11), otherwise the trypanosomes closely resembled *T. gambiense*. Another feature of the case was that only *Gl. morsitans*, which up till then had not been suspected of being capable of transmitting trypanosomiasis to man.

THE PANORAMA

TRYPANOSOMES

Travelled to the town of Pucallpa, to the patient had travelled South
Holland, a return of the disease was reported from the
coast of Lake Ayacucho, and it is now known that the disease prevails
in several other places of the Amazon in the adjacent parts of
the Bolivian Empire, Peru, and the Argentine Republic.

[illegible]

of *m. ratum* and not of *fulvipes* prevails. It was however shown by Kinghorn and Yorke, working on the Luangwa (a tributary of the Zambesi) that *tel. murina* could transmit trypanosomes from human cases to rats, the cycle in the fly being at least eleven days, and that a definite percentage of wild flies in this region harboured the human parasite. There is thus no doubt that man in widely extended regions of southern Central Africa is exposed

to danger when bitten by *Gl. morsitans*. As in the case of *T. gambiense* only a small proportion of flies fed upon an infected animal become infective. The infectivity of the trypanosome for the fly seems to depend in great measure on the species of the vertebrate host harbouring the trypanosomes on which the flies are fed (Corson). *T. rhodesiense* may sometimes be transmitted by tsetse flies other than *Gl. morsitans*.

It is generally accepted that *T. rhodesiense* is distinct from *T. gambiense*. The disease which the former causes in man tends to be more acute. Frequently there is not a terminal sleeping sickness stage—death occurring from visceral lesions before the central nervous system is involved in untreated cases, and there is less pronounced infection of lymphatic glands. In monkeys experimentally infected the trypanosomes multiply in the myocardium at a distance from the blood vessels and lead to myocarditis and pericarditis, also serous effusions (Peruzzi); and similar lesions occur in man. The organism is also more virulent for animals, the duration of the illness being shorter, and the susceptibility of the sheep and goat is greater than towards *T. gambiense*. In both of these animals widespread œdema, especially of the face, is a marked characteristic. *T. rhodesiense* infection in mice is influenced by human serum, which has no therapeutic effect on the infection with *T. gambiense* in these animals. There has been considerable controversy regarding the relationship of *T. rhodesiense* to *T. brucei*. The lack of infectivity of *T. brucei* for the human subject has been repeatedly proved by the inoculation of volunteers. But while differences in the pathogenic effects of the two organisms have been observed, the right interpretation of the data constitutes a difficult question. Bruce and his co-workers, founding largely on extended biometric investigations, were of opinion that the *T. rhodesiense* is a strain of *T. brucei* which has adapted itself to man. As mentioned above, the view is now generally accepted that *T. gambiense*, *T. rhodesiense*, and *T. brucei* are biological races of the same species.

LABORATORY DIAGNOSIS OF TRYPANOSOME FEVER AND SLEEPING SICKNESS

Trypanosomes should be looked for in fresh and stained films of the juice of lymph glands, blood, cerebro-spinal fluid, and bone marrow. In the case of the *cerebro-spinal fluid* (obtained either by lumbar or cisternal puncture) about 10 c.c. should be centrifuged for fifteen minutes and the deposit placed under a cover-glass for examination (to avoid injury to the delicate structure of the organism make a little cell on a slide by painting a ring of ordinary embedding paraffin, place the droplet of fluid in its centre and support the cover-glass on the paraffin). In fresh cerebro-spinal fluid the trypanosomes can be seen to be actively motile; the number in which they occur varies very much, and the same is true to a greater degree of the blood, in which they are, however, usually very scanty. With regard to the *blood*, fresh films or thin or thick stained films may be examined as in the case of malaria. For concentrating the organisms Bruce and Nabarro recommended that the blood be mixed with citrate of sodium solution (equal parts of blood and of a 1 per cent. citrate solution) and centrifuged for ten minutes, that the plasma be removed and centrifuged again for the same time, and that this be repeated three times, the deposit on each occasion after the first centrifuging being carefully examined. Examination of the *lymph glands* in a suspected case is a valuable means of diagnosis. The method is to puncture the gland with a hypodermic needle, suck up a little of the juice,

and expel it on to a slide.

Leishman's stain is difficult, but good preparations are obtained by the procedure originally recommended by Leishman for sections of cerebro-spinal fluid and gland.

Method After the section has been brought into distilled water, excess of the latter is blotted off, a drop of fresh blood serum is then placed on the preparation and allowed to soak in for five minutes. The excess is removed by blotting, and the remainder is allowed to dry on the section, which is now treated with a mixture of two parts of Leishman's stain and three of distilled water, and placed in a Petri dish for one to one and a half hours. Decolorisation and differentiation are effected by alternately applying dilute acetic acid and caustic soda solutions (Appendix).

The presence of scanty parasites may be shown by subcutaneous or intraperitoneal inoculation of a rat, mouse, or monkey with blood, cerebro-spinal fluid, etc from a suspected case. The animal's blood should then be examined at intervals of several days for the next month or two—in the case of a rat or mouse this is readily done by snipping a small piece off the skin of the tip of the tail, and expressing a drop of blood. It must be noted, however, that negative results have no significance, as an infection may fail to develop in the animal even when trypanosomes can be detected by microscopic examination in the inoculated blood. The occurrence of *autoagglutination of the red corpuscles* when a wet film of blood of a patient is examined between a slide and coverslip is highly suggestive of trypanosomiasis, although not diagnostic in itself.

TRYPANOSOMA CRUZI

A disease of man associated with trypanosome infection was originally discovered in Brazil, where Chagas observed the trypanosome in a monkey which had been bitten by a reduviid bug, *Triatoma megista* (*Panstrongylus megistus*), the latter harbouring numerous flagellates in its intestine. As these insects also feed on man both in their winged and nymph stages, the possible relationship of the trypanosome to a human disease in that region was investigated. The trypanosome has since been found to be much more widely distributed geographically, having been met with as far north as California in the rodent *Neotoma fuscipes*. Accordingly, the occurrence of the human disease appears to depend on a number of factors acting concurrently.

The disease affects children in various parts of S. America, and gives rise to marked fever, the occurrence of oedema (conspicuous in the face), and enlargement of the thyroid, lymphatic glands, the spleen, and liver. It may cause death in a few weeks, or assume a very chronic form which has been stated to be characterised by disorders of internal secretion—myxoedema, bronzing of skin, and infantilism—although the relation of these changes to the infection has been doubted. Cardiac and neurotropic forms have also been described. In adults the condition tends to be of the chronic form, or latent. The trypanosomes are readily found in the peripheral circulation in acute cases in the first ten to thirty days of illness (Fig 195, 12-14), but at the chronic stage they may not be detectable on microscopic examination, although they may persist for many years. As seen in the blood *T. cruzi* presents slender forms, which show active movements of progression, and larger forms which do not shift their place. The special feature of interest is the multiplication of the parasite, which does not occur in the blood but in the tissue cells. Thus *post mortem* in man, the parasite is found chiefly within

Not reported

T. congolense and *T. vivax* cause serious disease in cattle, horses, sheep and other domestic animals in Africa. *T. vivax* is distinguished by its active movements of progression, it measures 18 to 26 μ in length, has a definite flagellum and the macronucleus lies towards the anterior end of the body. In the tsetse fly (*Glossin morsitans* and other species) multiplication of the trypanosomes occurs only in the proboscis. *T. vivax*, but *T. vivax*.

T. evansi

found in Asia
camels. The

Stomoxys calcitrans it is a monomorphic trypanosome resembling *T. brucei*, though frequently shorter, a variety of experimental animals are susceptible to infection.

T. equiperdum is similar to *T. evansi*; the disease, Dourine, which it produces in horses resembles nagana in many ways. It is peculiar in that infection does not take place by an intermediate host, but directly through coitus, as it occurs only in stallions and in mares served by these. Laboratory animals are susceptible to experimental inoculation with blood containing the trypanosomes.

T. equinum infects horses in S. America, producing Mal de Caderas; other domestic animals are less severely affected. Biting flies are believed to transmit this infection. While generally like *T. evansi*, a striking feature of *T. equinum* is the minute size of the blepharoplast, and the parabasal body is absent. Laboratory rodents are susceptible to inoculation.

T. theileri, a trypanosome found in cattle in Africa and other parts of the world, is

five forms of the parasite develop. It does not infect other animals.

Trypanosoma lewisi. This trypanosome is very common in the blood of rats all over the world, though the percentage of animals affected varies in different localities. The

infection runs a very definite course in the rat, although usually without signs of serious illness and only very rarely fatal. In the later stage of the infection, which may last for several months, the parasites do not multiply to any considerable extent. They are actively motile, of ordinary length but somewhat narrow, the posterior end being pointed, the macronucleus lying in front of the centre of the body, while the protoplast does not contain any granules. In four to six days after intraperitoneal inoculation with blood from an infected rat the organisms begin to appear in the blood, and their rapid and very extensive multiplication occurs. At this stage the parasites show great pleomorphism. Finally the organisms gradually disappear from the blood. In the great majority of cases the rat is now immune against fresh infection. Trypanosomes introduced into its peritoneum are, according to Laveran, taken up by mononuclear phagocytes and destroyed. The serum of a rat which has been infected shows agglutinating action on the trypanosomes, causing them to agglomerate in rosettes in which the flagella are directed outwards; the serum of immune rats has also a certain degree of protective action if injected along with the organisms into a susceptible animal. According to Taliaferro, two distinct specific antibodies come into play in the course of the infection—

being often produced (the optimum temperature is about 20°C). These are not pathogenic when introduced into rats give rise to the usual infection. Novy and MacNeal succeeded in carrying a growth through many subcultures. The trypanosome is very resistant to cooling and has been exposed for fifteen minutes to the temperature of liquid air (-191°C) without being killed. Rabinowitch and Kempner have shown that the rat *Rattus norvegicus*, *fasciatus*, transmits the parasite, infection occurring through the fleas or their feces being

swallowed (Noller, Minchin and Thomson, and others). In the flea a phase of multiplication of the trypanosomes occurs within the epithelial cells of the stomach, the parasites then migrate to the hind-gut and rectum, assume the crithidial form and undergo fission, and finally take on the trypanosome form again. The flea becomes infective about a week after biting, and remains infective for the rest of its life. Infection may also take place through other species of fleas and through the rat louse (*Hæmatopinus spinulosus*).

Similar trypanosomes occur in other rodents, each being apparently adapted to its specific host, e.g. *T. nabiasi* (*cuniculi*) in the rabbit, *T. duttoni* in the mouse, etc.

CHAPTER XXXVI

PATHOGENIC PROTOZOA (contd.)—LEISHMANIA : TOXOPLASMA

LEISHMANIA

THESE parasites, which are classed in the same family as the trypanosomes, cause three human diseases. Although microscopically indistinguishable, it is generally held that the organisms belong to two or possibly three distinct species—*Leishmania donovani* (the name given to it by Ross), associated with the human disease kala-azar; *Leishmania infantum*, producing a similar disease in children (possibly identical with *L. donovani*); and *Leishmania tropica*, which has been found in a skin ulceration of widespread geographical distribution. The leishmania body, which is best demonstrated by a Romanowsky stain, e.g. Leishman's, is round, oval, or cockleshell-shaped, and usually 2.5 to 3.5 μ in diameter, though smaller forms occur. The protoplasm stains pink, or sometimes slightly bluish, and contains two bodies taking on the bright red colour of nuclear chromatin. The larger (trichonucleus), which stains less intensely than the smaller, is round, oval, heart-shaped, or bilobed, and lies rather towards the periphery of the body—in the region of the 'hinge' in the cockle-shaped individuals. The other chromatin body (kinetocore or kinetoplast) is usually rod-shaped, and is set perpendicularly or at a tangent to the larger mass, with which only exceptionally it appears to be connected. According to Christophers, Shortt and Barraud, the smaller chromatin body is differentiated into the parabasal body, and, running at right angles to the long axis of the latter, the rhizoplast (axoneme), which is a straight or slightly curved linear structure measuring about half the diameter of the parasite in length. The aspect of the parasite on which the parabasal body is situated is taken to be dorsal, and from it, at a point corresponding to the blepharoplast, the rhizoplast extends anteriorly. Usually the protoplasm contains one or two vacuoles. The bodies are found chiefly within the protoplasm of macrophages where they have multiplied, but they may also occur free, usually as the result of bursting of such cells. It has now been established that the parasite passes another phase of its existence in blood-sucking flies of the *Phlebotomus* genus (sand flies). In the alimentary tract of the insects a leptomonas form is produced, i.e. a trypanosome-like structure devoid of an undulating membrane and with the flagellum becoming free at the end where the micronucleus is situated; these proliferate. A fly becomes infective naturally if the leptomonas forms invade the distal part of its epipharynx and are ejected when it bites. Man is not the only mammal susceptible to infection with leishmaniae. Dogs are found to be infected naturally in certain areas, e.g. on the Mediterranean coast, and there is good evidence that they constitute the reservoir whence children acquire the disease in that region. On the other hand, the association of leishmania infection in the dog and the human subject is by no means invariable. Various rodents and other mammals can be infected experimentally with leishmaniae, and certain rodents which are naturally infected, have been shown to be the source of cutaneous leishmaniasis in some countries.

Leishmania donovani. Leishman noticed in several soldiers invalided from India for remittent fever and cachexia that very careful examination of

the blood failed to reveal the presence of malaria parasites. Most of these patients during their service had been quartered at Dum-Dum, an unhealthy cantonment near Calcutta, and from this fact the existence of a disease hitherto undescribed was suspected. In 1900 Leishman found in the spleen of such a case peculiar bodies, which resembled degenerating forms of *T. brucei*, and he suggested that they might be trypanosomes. Leishman's observations were confirmed in India by Donovan, and the bodies associated with the disease are now usually called the 'Leishman' or the 'Leishman-Donovan' bodies. They were found by Bentley, and later by Rogers, in the disease known in Assam as kala-azar, the pathology of which had long been obscure, since, while it resembled malaria in many ways, no malaria parasites could be demonstrated. This disease has gone under various synonyms, e.g. cachectic fever, Dum-Dum fever, non-malarial remittent fever, but is now recognised as a single specific entity—visceral leishmaniasis.

Kala-azar (or 'black disease'—so called from the hue assumed by chocolate-coloured patients suffering from it) has been known since 1869 as a serious epidemic disease in Assam, where it has spread from village to village up the Brahmaputra valley. The disease occurs in many parts of India, China, Turkestan, the Malay Archipelago, North Africa, the Sudan, Syria, Arabia, and also South America (see Senekjic). The disease is characterised by fever of a very irregular type, by progressive cachexia, and by anæmia associated with enlargement of the spleen and liver. Some cases at the commencement may resemble enteric fever. Rogers pointed out that there occurs a leucopenia which is almost always more marked than that of malaria—the leucocytes usually numbering less than 2,000—further, the white cells are reduced in greater ratio than the red corpuscles, which condition, again, does not occur in malaria. The disease is chronic, often going on for several years, and in, at any rate, 80 per cent. of the cases has a fatal issue unless treated by antimony compounds or diamidino stilbene. It is of interest in connection with the cutaneous disease caused by *L. tropica* (vide *infra*), that in kala-azar ulcers of the skin may occur. Also, Brahmachari and others have recorded the development of skin nodules containing the parasites, as a late form of recurrence in patients who had not been completely sterilised by treatment, but in whom visceral infection did not recur (see Kirk).

Post mortem, there is little to note beyond the enlargement of the liver and spleen, but in the intestine, especially in the colon, there are often large or small ulcers, and there is evidence of proliferation in the bone marrow, the red marrow encroaching on the yellow. The histological changes consist essentially in an increase of the large mononuclear phagocytes (see Meleney).

In a smear made from the spleen and stained by Leishman's solution the characteristic bodies can be readily demonstrated (Fig 197). Although in such films many free bodies are seen, the study of sections shows that ordinarily their position is within the protoplasm of large mononuclear cells—macrophages. The view held is that on their entering the circulation they are taken up by the mononuclear leucocytes and by such cells as the endothelial lining of the splenic sinuses or those lining capillaries or lymphatics, that in these cells multiplication takes place—it may be to such an extent as to rupture the cell—and that if thus the bodies become free they are taken up by other cells and the process is repeated. The clusters of bodies sometimes seen in smears are probably held together by the remains of ruptured phagocytes. In capillaries the endothelial cells after ingesting the bodies probably become detached from the capillary wall, as they are often observed free in the lumen of the vessel—this being well seen in the

hepatic capillaries. The parasites are found in greatest abundance in the spleen, liver, and bone marrow, and also in mesenteric glands, especially in those draining one of the intestinal ulcers; less frequently they occur in the skin ulcers, and in other parts of the body, *e.g.* the villi of the intestine may be crowded with them (Perry). Donovan described them as occurring in the peripheral blood, especially within the leucocytes, and this observation has been generally confirmed, though sometimes prolonged search is necessary. Patton has found that the numbers in the blood vary from time to time, and special incursions may be associated with exacerbations of dysenteric symptoms which, he holds, indicate a spread of the intestinal ulceration. Shortt and his co-workers frequently recovered the organisms in cultures made from the urinary sediment. They occur also in the secretions of the mouth and nose (Forkner *et al.*).

FIG. 197. Leishman-Donovan bodies from spleen smear, some are within the endothelial cells. Leishman's stain $\times 1,000$.

being formed, but sometimes a multiple division takes place, each nucleus dividing several times within the protoplasm and a corresponding number of new parasites resulting.

CULTIVATION Rogers made the important discovery that in cultures a flagellate organism developed from the Leishman-Donovan body. Thus Leishman's original view on the relation of leishmania to the trypanosomes was confirmed. Cultivation was effected by taking spleen juice containing the parasite, placing it in sodium citrate solution, and keeping it at 17° to 24° C. Under such conditions there occurs an enlargement of the organism, but especially of the larger nucleus. This is followed by the appearance of a pink-staining vacuole in the neighbourhood of the smaller nucleus. Along with these changes, in from twenty-four to forty-eight hours, the parasite becomes elongated and the smaller nucleus and its vacuole move to one end, from the vacuole there then appears to develop a red-staining flagellum, which when fully formed seems to take its origin from the neighbourhood of the small nucleus. The body of the parasite is now from 20 to 22 μ long and 3 to 4 μ broad, with the flagellum about 22 μ long. The whole development occupies about ninety-six hours. The formation of an undulating membrane was not observed, and, although the flagellated organism moved flagellum first, like a trypanosome, it is evident that here the relationship of the micronucleus is different, as this structure lies anterior to the macronucleus. The serum of many animals, *e.g.* man, guinea-pig, has an inhibitory effect on the parasite, but success in the cultivation has attended the use of Novy and MacNeal's medium made up with rabbit's blood (that of the sheep or dog may also be used).

In the body the parasite multiplies by simple fission, both nuclei dividing amitotically and two new individuals

FIG. 198. *Leishmania donovani* from culture. Leishman's stain. $\times 1,000$.

The following procedure has been recommended as improving the medium for cultivation of leishmaniae. To each tube of 18 mm diameter containing 9 c c 2 per cent melted agar medium 3 c c defibrinated rabbit blood are added, and the tubes are kept in the sloped position at room temperature for twenty-four hours. They are then capped and placed at 37° C for twenty-four hours. Thereafter 2 c c 0.85 per cent NaCl solution are added to each tube and they are capped again and placed in a sloping position at 37° C for twenty-four hours further. Growth is richest toward the surface.

Lourie has used the following simple semi-solid medium both for primary culture of leishmaniae and for maintaining subcultures. 0.3 gm of powdered agar is dissolved in 100 c c saline by raising to the boil in a flask and boiling for 2 minutes. Then the flask is cooled in a water-bath to 45°-50° C and 15 c c sterile defibrinated rabbit's blood is added. This is well mixed and amounts of 3 to 15 c c are placed in 15 cm by 1.5 to 1.75 cm tubes, these are not plugged but are covered with glass caps. The medium keeps in the ice chest for many months. Cultures kept at 15° to 21° C remain viable for at least 2 or 3 months. Growth is more rapid at 22° to 25° C.

The phases of the parasite in such cultures have been investigated by Christophers, Shortt and Barraud, they concluded that the masses of mature flagellate forms which develop from the Leishman-Donovan bodies, leave the group where they were produced and become free-swimming. These separate and later come to rest; then as a result of repeated divisions each gives rise to a number of short multiplicative forms, some of which again are provided with vibratile flagella and, becoming free-swimming, repeat the cycle. In cultures which have been kept some time (at least seven days), small rounded non-flagellate forms appear which measure about 2 μ , these are considered by Row to be of the nature of cysts, but have been regarded by others as degenerate forms. The relative infectivity of the leptomonas and the rounded (leishmaniod) forms has not been settled (see Southwell *et al*). In old cultures Leishman described the occurrence of unequal division of the parasite, which resulted in the splitting off of a hair-like undulating form containing a chromatin granule, the final development of these spuriary forms has not been traced.

The cultivation of leishmaniae in the yolk sac of the developing hen's egg has also been effected (Jones *et al*).

Experimental Infections. Though results obtained in different parts of the world vary somewhat, certain animals (*e.g.* monkeys, young dogs, and mice) have, in a varying proportion of cases, been infected with the parasite as it occurs in human lesions and also in cultures. The intraperitoneal route is the best, and both when the animals have died and have been killed leishmaniae have been found in such situations as the spleen, the liver, and bone marrow. Recovery from the infection is followed by immunity to reinoculation. Feeding experiments have usually been unsuccessful, but some positive results are recorded. The observation of Smyly and Young that *Cricetus griseus* (the Chinese hamster) and other hamsters are highly susceptible to intraperitoneal inoculation has greatly facilitated such investigations. Infection has also been produced, in descending order of certainty, by the subcutaneous, oral, percutaneous, and conjunctival routes. Brumpt and Gaillard have found the spermophile (*Citellus citellus*) susceptible in the case of a Chinese strain of the parasite. The infection in animals tends to have a chronic course.

Natural Mode of Transmission. The question arises, given that *L. donovani* is the cause of kala-azar, how is infection spread? Various theories have been held. The excretion by patients of living parasites, their survival in a moist state for several days, and positive results in feeding experiments suggested that the infection may be spread by such means. The relationship of the organism to the trypanosomes and its presence in the blood made it appear likely that a blood sucking insect might act as the intermediary host. The bed bug (*Cimex rotundatus*) was suspected, but it was shown that bugs

obtained from the bedding of kala-azar patients, or fed upon such cases, very rarely contained flagellates, and material from such bugs when injected into monkeys did not produce the infection. As regards other insects, a frequent difficulty has been the presence of a variety of flagellates which normally inhabit their intestines. The Kala-azar Commission, however, brought forward strong evidence that a sandfly, *Phlebotomus argentipes*, is the insect host, since its distribution corresponds with that of kala-azar. When flies of this species were fed upon cases of the disease, flagellates appeared in the intestine of a high proportion (Knowles, Napier and Smith; and others). In view of the scantiness of leishmaniae in the peripheral blood of patients, such multiplication in this fly indicates that it affords a specially favourable medium for the parasite. The appearances met with in the intestinal contents of the infected flies were the same as those found in artificial cultures of leishmaniae; further, a large number of flies fed upon healthy individuals failed to show flagellates. It is of importance also that in several instances the Commission carried out control experiments in which the same cases as caused infection of *P. argentipes* were fed upon by bugs, but with entirely negative results. The *Phlebotomus* flies showed a marked infection of the gut five days after feeding upon a case, and in a considerable proportion of flies which survived to the eighth day the pharynx and buccal cavity contained flagellates, but they did not become established to any extent in the rectum. In connection with the identification of insect hosts of the leishmaniae, it is noteworthy that these protozoa will develop only in the absence of bacteria, this condition is realised in the alimentary tract of *Phlebotomus* flies. As regards capacity to transmit the disease, it is only in certain species of these flies that the proliferated leishmaniae make their way to the proboscis, whence apparently the parasites are conveyed to the human host by the fly in the act of biting. However, the transmission of the infection to the Chinese hamster by repeated bites of artificially infected *Phlebotomus argentipes* was only occasionally successful (see Shortt, 1915-46), and attempts to transmit the infection to human subjects were unsuccessful, until it appeared that the mode of feeding the infected flies might be a determining factor. If flies which have ingested infected blood receive meals of blood subsequently, the development of virulent leishmaniae is not favoured; but if they are fed on sugary matter (raisins) there is a much greater proliferation of the parasites and these come to 'block' the pharynx (this explanation, however, has not been generally accepted). Infected flies treated in this way have transmitted the parasites readily both to man and animals (Smith *et al.*, Swaminath *et al.*). It is also possible, however, that different strains of *L. donovani* may vary in their infectivity. Other factors which may influence the infectivity of the flies are discussed in connection with *L. tropica* (p. 693). Complete development of the parasite occurs also in *P. chinensis* and this species is regarded as the transmitter in China. In other parts of the world different species of *Phlebotomus* flies are responsible. The possibility that infection is received from the fly otherwise than by its bite has not gained much support.

The question arises as to how far other mammals may be a source of infection for man. While there is strong evidence that in China dogs are an important source of the disease, in India the examination of dogs from the neighbourhood of kala-azar cases has not yielded evidence that natural infection occurs in these animals. The part played by infected dogs is discussed further under *L. infantum*. Cats and other domestic animals have also been stated to be infected; but there is no evidence that they are of importance in relation to the human disease.

Leishmania infantum. Nicolle, working in Tunis, observed a disease clinically identical with kala-azar, usually affecting children between two and five years of age, and exceptional after the age of fifteen. He found in the spleen, liver, and bone marrow in such cases an organism microscopically indistinguishable from the *L. donovani*. Cutaneous and intestinal ulceration, such as are met with in kala-azar, do not occur. The disease, although sporadic, is very widespread, and occurs along the whole of the south and east littorals of the Mediterranean, also in Spain, Portugal, Greece, Sicily, France (Côte d'Azur), and in Italy as far north as Rome, in the Sudan, and Abyssinia.

The organism can be successfully inoculated in the dog, monkey, mouse, rat, guinea-pig, and rabbit by intrahepatic or intraperitoneal injection of spleen pulp from fatal human cases. Also, several species of hamsters have proved susceptible to inoculation with the organisms from tissues or cultures (Adler and Theodor, and others).

The organism has been regarded as a separate species. In support of this is the fact that the disease, as it occurs in the regions named, is apparently confined to young children. Nicolle considered the infection of the dog to be significant, as this animal might be the channel through which children became infected, for in most regions where the disease prevails there occurs a disease of dogs, which may be either of an acute or chronic character, and which is apparently due to an identical organism. In infected dogs ulcers may occur in the skin, and both in these and also in apparently healthy areas of skin, numerous leishmaniae are present. On the other hand, Laveran found that a *Macacus* monkey which had recovered from an infection with leishmania derived from Tunis, was also immune to inoculation with parasites obtained from a case of Indian kala-azar, thus it would appear likely that the two organisms are identical, although they show certain differences (vide p. 695). Serological reactions are also in favour of the identity of the two. The evidence points to *Phlebotomus perniciosus* being the important vector (Adler and Theodor), but other *Phlebotomus* flies may also be vectors, e.g. *P. major*.

Leishmania tropica. In various tropical and sub-tropical regions (India, Middle Asia, Greece, Italy, Spain, Egypt, Northern Africa, the Sudan, Turkey, South America, West Indies) there is widely prevalent a variety of very intractable chronic ulceration which goes by various names in different parts of the world—Delhi sore, tropical ulcer, Aleppo boil, etc. These sores at the commencement may be single or multiple—they may spread from one part of the body to another by auto-inoculation, also they may be communicated to other individuals by contact. Wright, of Boston, U.S.A., first showed that a protozoal parasite microscopically indistinguishable from *L. donovani*, is concerned in the etiology of the condition. Great numbers of leishmania bodies were demonstrated in the discharge from the ulcer and in sections of a portion of tissue excised from a case coming from Armenia. It was found that the bodies were usually intracellular in position in the lesion, as many as twenty being in one cell, and that the type of cell containing them was, as in kala-azar, the macrophage (Fig. 199). These observations have been fully confirmed by workers in various parts of the world. The duration of the sore is about a year. After recovery the individual possesses immunity to re-inoculation. Sometimes the parasite is destroyed in a foul ulcer, but can still be obtained by taking some of the juice from the marginal indurated tissues by capillary glass tubes. Patton reported finding the organism in blood taken from parts adjacent to the ulcer.

The organism may be grown in Novy and MacNeal's medium or in

citratated blood. The lesions were identical with those naturally occurring, the incubation period being often many months. The organism may still be pathogenic for man after cultivation for four and a half years. In tissue cultures the leishmania forms are produced (Weinman). Animals can be infected experimentally, e.g. monkeys, dogs, and various rodents. In the monkey the lesions are identical either by a granuloma in the skin or around the joints; all these lesions contain numerous parasites. Cutaneous leishmaniasis occurs in dogs, and Adler and Theodor have produced a typical skin lesion in the human subject by inoculation with a culture of canine origin.

Evidence has been furnished that the disease is transmitted by *Phlebotomus* flies. Adler and Theodor in Palestine inoculated into the skin in human subjects the contents of the alimentary tract of *P. papatasi* which had been fed on lesions at least eight days previously and contained numerous leishmanias forms; from seventeen to thirty-three days later a small papule developed at the site of inoculation in a third of those inoculated, which contained leishmanias. Numerous attempts to transmit the infection by the bites of the flies proved unsuccessful. Adler and Ber came to the conclusion that intensity of infection in the fly was not the most important factor, but that the distal part of the epipharynx must be infected before the leishmanias forms were deposited on the skin in the act of biting. By feeding *Phlebotomus papatasi* with infected blood



FIG 199. *Leishmania tropica* Smear from sore showing macrophage containing numerous leishmania bodies. Leishman's stain $\times 1,000$

mixed with hypertonic (2.7 per cent) saline and keeping them at 30° C., they found that the flies became ready transmitters of the lesions to human volunteers. (It is possible of course that the strain of *L. tropica* used was a readily transmissible one.) Other species of *Phlebotomus* flies may also act as the vectors.

Russian workers in Middle Asia (Russian Turkestan) have recently shown (see Hoare) that the two clinical types of lesion, also noted by others, the 'dry' and the 'moist' (i.e. rapidly ulcerating), are due to distinct strains of the parasite. The evidence for this is that on experimental transmission, either directly or by means of cultures, the lesions remain of the same type, further, immunisation with the one form does not confer immunity to the other. It has been established also that the parasite of the moist type, which is the prevalent one in desert country districts, exists naturally in rodents, especially gerbils, in these animals it produces a very chronic disease. *Phlebotomus* flies (*P. papatasi* and *P. caucasicus*) which breed in the burrows of the rodents, transmit the infection—the former to man, the latter chiefly among the rodents.

In the South American form of the disease, which is found especially in forest areas, after the skin ulcers have appeared, typical lesions occur not uncommonly at a later date in the nasal and buccal mucous membranes, the so-called muco-cutaneous form, such cases are liable to develop a severe

cachexia. It has been suggested that this condition is due to a specific parasite, *L. brasiliensis*. Dogs are affected as well as man. Phlebotomus flies are probably the vectors.

Differentiation of the Leishmaniae. The close similarities in the morphology and effects of the leishmaniae described above naturally raise the question whether we are not dealing with variants of one organism whose differences depend on differences in the virulence of different types or on the susceptibility of different hosts. The following are some of the facts bearing on this question. The Indian and Mediterranean diseases are apparently identical clinically, although the age incidence is different. But in certain parts of India kala azar is chiefly found in children below the age of fifteen while, on the other hand, in regions where the infantile variety prevails cases occur in young adults. The importance of such factors as racial susceptibility is indicated by the fact that in Tunis it is chiefly the children of Italian parentage who suffer. Kala-azar and Oriental sore are linked by the occurrence in the former from time to time of skin ulcers, although in these—unlike the case of Oriental sore, the parasites are difficult to find. On the other side it is of importance that cases of Oriental sore do not develop into kala azar. But in dogs inoculation with *L. donovani* may cause skin lesions resembling those caused by *L. tropica* in man (Campanopetros). Agun Nicolle found that dogs infested with *L. tropica* appeared to have an increased resistance to *L. infantum*. The incidence of canine leishmaniasis in communities where a human infection prevails varies in different regions, e.g. even in the Mediterranean littoral, though it is usually common where *L. infantum* is found. But dogs are extensively infested in Turkestan where the human incidence of the disease is among adults. The problem of the relationships of the leishmanae has been investigated by serological methods (Noguchi, Khigler, Adler and Theodor). It has been found that antisera obtained from rabbits by injecting intravenously cultures of the organisms possess both agglutinating and lytic action. The results obtained by the use of such sera indicate that *L. infantum*, *L. tropica* and *L. brasiliensis* are specific organisms, on the other hand *L. donovani* and *L. infantum* are indistinguishable. But there appears to be a certain amount of group reaction among the organisms. It is more typical, e.g. in the Sudan, cutaneous disease, visceral leishmaniasis and mucocutaneous forms of leishmaniasis occur together (Kirk). It is not possible to decide whether this is due to the existence of strains of leishmania which differ in virulence and tissue affinity, or whether it results from a single strain in hosts of varying susceptibility. But the facts that all three forms of the disease may follow a more or less identical path in the same patient and also that when the disease occurs in a district over a period of time the manifestations tend to differ from the visceral to the cutaneous type suggest that when the defence against the infection is differently vigorous it permits of the development of a different type of parasitic establishment. There is considerable evidence that these represent the localised, tertiary distribution of the infection. Nag and Khigler (Kirk), Ascher and Adler (Kirk) and the Sudan Committee (Kirk) have shown that the localised tertiary lesions caused by *L. infantum* and *L. donovani* have a resistance to *L. infantum* and *L. donovani*. H. J. Wilson has shown that the localised tertiary lesions caused by *L. tropica* have a resistance to *L. tropica* and *L. infantum*.

Latent or Dormant Leishmaniasis. The leishmanae have been shown to persist in the cells of the reticulo-endothelial system of the infected animal. The cells are not fully transformed into the amastigote form, the leishmanae are not fully developed and do not reproduce. It is probable that the leishmanae in this condition are not infective to other animals.

since hæmorrhage from this organ is liable to occur; liver-puncture is less dangerous, but the parasites are not so readily found in this way; the juice of enlarged lymphatic glands should also be examined. A perfectly dry needle must be used for obtaining the juice of organs, as traces of moisture cause distortion of the parasites. The parasites may also be stained in sections (for method see p. 801). The microscopic demonstration of the parasites may be attempted by means of films

made on slides, the thick edge should be examined specially, since the parasites are usually contained in the leucocytes which accumulate at this part of the film. Another method consists in inoculating a number of tubes of Novy and MacNeal's or Lourie's medium each with several drops of blood from the patient; or in adding 2.5 c.c. of blood aseptically to 10 c.c. citrate and salt solution (p. 801), sedimenting the corpuscles, and then by means of a pipette inoculating tubes of the medium with the sediment. The cultures should be kept at 22° to 26° C. and examined after a few days, and thereafter for two or three weeks. After specific treatment the demonstration of leishmaniasis is much more difficult. Leucopenia and the absence of malarial parasites from the blood are also important features. The complement-fixation reaction has been obtained with the patient's serum and an antigen composed of suspensions of cultures or of heavily infected organs from experimentally inoculated animals. Allergic skin reactions with killed cultures have also been described. Napier's 'Aldehyde reaction' consists in the addition of one drop of formalin to 1 c.c. of blood serum with thorough mixing; when the serum is derived from a fully developed case of kala-azar the mixture in a few minutes resembles boiled egg-white. This result, at least in a partial form, may, however, be obtained with serum from other conditions; also in some cases the reaction may be negative. A similar reaction has been got with a pentavalent antimony compound as the reagent. A complement-fixation reaction with the serum in kala-azar and an antigen derived from tubercle bacilli is stated to be highly specific (see Sen Gupta; Shortt, 1947).

In cutaneous lesions the parasites are demonstrated in scrapings from the ulcers. But if there is also bacterial infection, the leishmaniasis will be more readily found by puncturing the tissue near the margin of the ulcer (Care must be taken not to mistake yeast-like organisms for leishmaniasis).

TOXOPLASMA

This organism, which is believed to be a protozoon, occurs in various animals including dogs, but especially in rodents and birds, and has a wide geographical distribution. It has also been described rarely in diseases of man. As seen in smears from tissues it is crescentic, but often with one end more rounded than the other. There is a nucleus which is central or nearer to the rounded end. The appearance resembles that of a leishmania but the size is greater, 4 to 6 μ by 2 to 3 μ , and there is no definite blepharoplast. It is well stained by hæmatoxylin or by Giemsa's method and is Gram-negative. In sections of tissues the parasites are more rounded. They occur in masses within cells and may resemble cysts. Their mode of reproduction is believed to be by longitudinal division. In susceptible animals experimental inoculation with a virulent strain leads to death within a few days to some weeks. Lesions of encephalitis are a feature especially after intracerebral inoculation. In man these organisms have been found in adults suffering from a typhus-like disease (Pinkerton *et al.*) and a condition resembling infective mononucleosis (q.v.) and in infants and young children with the signs of meningo-encephalitis (q.v.). *Post mortem* the parasites are detected in various viscera. The natural mode of transmission is not known. Diagnosis may be made by microscopic examination of smears and sections of tissues and the centrifuged sediment of the cerebro-spinal fluid, as well as the sputum. Also, animals should be inoculated, several

rice and guinea pigs being injected intracerebrally and intraperitoneally with a suspension of tissue which must be fresh ground in a mortar (without an abrasive) or with the sediment from cerebro-spinal fluid. The brain and lungs as also any peritoneal exudate of inoculated animals should be examined for the parasites. The organisms may not have high virulence in the first passage, but this may be increased by passages in fresh animals. Care must be taken to ensure that the stock of animals used does not have a latent toxoplasma infection, which may be activated in this way. The serum of certain human cases contains antibody which when added to the toxoplasma neutralises the local pathogenic action of the latter on intracutaneous injection in rabbits. Salmons

CHAPTER XXXVII

CHEMOTHERAPY

A CHEMOTHERAPEUTIC agent is a substance of defined chemical constitution which conduces to the cure of an infection by assisting the body in overcoming the causal organism—tonics which support the physiological functions of vital organs are, of course, not considered under this head. The oldest known chemotherapeutic agents are those which act in protozoal and spirochætal infections—quinine in malaria, mercury in syphilis, and ipecacuanha in amoebic dysentery. Only comparatively recently, from the stimulus of Ehrlich's work, has there been a systematic search for such drugs, the first synthetic compound being trypan-red (Ehrlich and Shiga), a single dose of which, when given to mice, cures a fatal infection with *T. equinum*, the trypanosome of a disease of horses in S. America. The first compound shown to act on a systemic bacterial infection was optoquine (an analogue of quinine), with which Morgenroth cured pneumococcus infection in mice. A number of drugs have now been found which act on various infections. In the quest for chemotherapeutic agents renewed attention has recently been directed to earlier observations that in mixed cultures certain organisms inhibit the growth of others and may lyse them. This property of *B. pyocyaneus* has been long known; and it was observed by Gratia and Dath that a penicillium and a streptothrix also acted similarly. The biological formation of various chemical products of defined constitution has been determined, e.g. penicillin, which is a mixture of several analogues, from a culture of *Penicillium notatum*, discovered by Fleming, and streptomycin, recovered from growths of *Actinomyces griseus* by Schatz, Bugie and Waksman. Penicillin was the first outstanding compound of this class because of its powerful action *in vivo* on certain Gram-positive pathogenic organisms, such as the staphylococcus, associated with extremely low toxicity for mammals (Chain and Florey). Streptomycin too combines marked chemotherapeutic activity with low toxicity, it acts also on Gram-negative bacilli, e.g. *B. pestis*, and on the tubercle bacillus. *Aerosporin*, a basic peptide formed by *B. aerosporus* (*B. polymyxa*), a sporing bacillus found in air and soil, has powerful bactericidal properties which are selective for Gram-negative organisms (Brownlee and Bushby). A large number of such compounds are now known which are produced by living organisms, but have not as yet been synthesised in the laboratory, they are spoken of as 'antibiotics' (p. 31).

In some cases it has been found that enzymes are produced which interfere with vital activities of other organisms, but need not actually kill them. For example, Dubos and Avery obtained from the growth of an aerobic sporing soil bacillus *in vitro* an enzyme which decomposed specifically the polysaccharide of the capsule of type III pneumococci. On the addition of the enzyme these organisms were deprived of their virulence, which depends on the aggressive action of the polysaccharide; and by injecting the enzyme intravenously into monkeys suffering from pneumonia due to type III pneumococci a marked curative action was produced (Francis *et al.*)

Difficulties attending chemotherapeutic investigations on the chemical side are that the action of any given compound is usually restricted to a particular organism or to a small group of related organisms, and also that very few general relationships have so far been discovered between chemical con-

stitution and therapeutic action. On the biological side certain principles of wide application have been established. Experimental results will be chiefly considered here, clinical applications being only touched upon. General methods of investigation will be discussed first, then the biological phenomena of the host-drug-parasite interaction and the mode of action of drugs on organisms. Lastly, the different classes of infective agents will be dealt with.

Methods of Chemotherapeutic Investigation

Experimental infections. The general procedure with any pathogenic agent is first to find a host which shows a fairly uniform behaviour when infected. Small animals are preferable to start with on account of economy and convenience in handling the large numbers required. In the case of organisms which produce a general infection, the inoculum is selected of sufficient amount and virulence to cause a fatal septicæmia in untreated animals. Where the infection tends to run a chronic course or to become

tained organisms are studied or the influence on the animal's general condition caused by the production and absorption of toxins.

Estimation of toxicity of drugs. The toxicity of the drug must be ascertained for normal animals in respect both of the route of administration (*e.g.* subcutaneous, intravenous, intracardiac, intraperitoneal or oral) and the number and spacing of the doses. Watch should be kept for both local and general pathological effects due to poisonous actions of the drug, and the possibility of cumulative effects and of late or chronic lesions should be borne in mind.

Therapeutic action. After the toxicity of the drug has been ascertained, therapeutic effects in *infected animals* may then be investigated. At the same time a sufficient number of similarly inoculated animals should be left untreated to serve as controls. A drug can be examined for curative action by beginning administration after inoculation, or for prophylactic effects by reversing this order. The most effective time for commencing treatment should be ascertained, as this may vary according both to the infection and the drug. Treated animals should be observed for a long time and the blood and tissues should be examined for the pathogenic organisms, since treatment, although failing to effect sterilisation, may convert a rapidly fatal infection into a chronic or latent condition. The efficacy of a drug may be represented by the ratio of the maximum dose tolerated to the smallest dose which produces cure of the infection under the same conditions of administration, etc. This is spoken of as the 'therapeutic index'. Accordingly, the toxicity of the drug for the host is always the limiting factor for a chemotherapeutic agent. Excessive doses may, by poisoning the host, favour the infection ('contrary effect').

Action of the drug on the organisms *in vitro* should also be examined, but it must be remembered that a powerful lethal effect in the test-tube need not be accompanied by similar activity *in vivo*; this is well shown in the case of *Bor recurrentis* and methylene blue. On the other hand, compounds may appear to have relatively little action on the parasites in the test-tube and yet be efficient chemotherapeutic agents. There may be several reasons for this: either the drug may be converted into an active derivative *in vivo* or it may render the parasites more vulnerable to the natural tissue defences,

although not directly killed by the drug. Among the organic trypanocidal arsenicals the pentavalent arsonic acids, *e.g.* tryparsamide, and the trivalent arsenobenzols, *e.g.* salvarsan, are both believed to become converted *in vivo* into the corresponding arsenoxides, which represent an intermediate phase to which the former is reduced and the latter oxidised. The basis for this view is that the arsenoxides are more actively trypanocidal *in vitro* than the corresponding compounds of the other two classes. Drugs like trypan-red and suramin are at most only weakly or slowly lethal *in vitro* for the trypanosomes which they influence *in vivo*, and they do not appear to undergo change into more active derivatives in the body. Their possible mode of action is discussed later. Prontosil (Domagk) is decomposed in the body with the liberation of sulphanilamide (Trefouël *et al.*), which itself is only slightly bacteriostatic *in vitro*, *e.g.* for streptococci. According to Burton, McLeod, and their associates, it may be the corresponding oxidation product, the hydroxyl-amino derivative, which is effective *in vivo*. Again, in the case of penicillin there is a powerful direct action on susceptible organisms both *in vitro* and in the living animal. Thus, certain species of organisms are readily killed by penicillin while they are actively growing, although in the resting state they are insensitive 'persisters' (Bigger).

Conditions for general action Where a drug is required to reach the organisms by way of the blood or lymph streams it may fail in the body though active *in vitro*. Thus, certain tissues may fix or destroy it and so leave too low a concentration free to act on the parasites. Again, either absorption, say from the subcutaneous tissue, may be too slow or excretion of the drug may be too rapid for effective action on the infecting organisms. The rate of excretion of a water-soluble compound may be slowed down by injecting it into the tissue as an emulsion in an oily vehicle, which produces a 'depot'. Of course, where a drug is excreted through a particular channel, *e.g.* by the kidneys or liver, it is possible to concentrate its action on the organs of the excretory system concerned. Similarly, when attempting to treat infections of the central nervous system by systemic administration it is essential that the drug should pass from the capillaries into the nervous tissue.

Conditions for local action With substances which are not altered in the body, but which tend to be fixed by the tissues, the effects *in vivo* and *in vitro* are most likely to correspond closely if the drug can be brought into intimate contact with the infecting organisms in a particular area, *e.g.* 'surface antiseptics' applied to infected wounds. Even here the action of the tissues and the proteins of serous exudates or pus in absorbing the drug and so diverting it from the organisms has to be considered.

Biological Phenomena of the Host-Drug-Parasite Interaction

Theoretically a chemotherapeutic agent might act exclusively on the host, either by stimulating its defences or by rendering it indifferent to the parasites. But in most cases the evidence shows a direct effect produced by the drug on the parasites, complete destruction of the organisms, such as might be about by a germicide acting *in vitro*—an internal antiseptics, as it imaged by the destroyed, act as often conduces therapeutic effect to the cure. Accordingly, it is obvious depends on various factors on the part both of parasite and host; and, since both are living agents, they may undergo changes which assist or impede

sterilisation Where the process of sterilisation takes place gradually or is incomplete an analysis of the factors is to some extent possible Several striking biological phenomena have been brought to light—acquired drug-resistance, serological resistance (serum-fast or relapse strains), therapeutic potentiation and interference, defence and immunity mechanisms An understanding of those phenomena, which were elucidated practically in the above order, is required for a discussion of the mode of action of chemotherapeutic agents

Acquired Drug-Resistance and other Factors in the Parasite which modify Sensitiveness to a Therapeutic Agent

It might be expected that apart altogether from previous contact with drugs, different strains of the same species of parasite would show marked differences in their susceptibility to chemotherapeutic agents It is not known whether this has the same basis as acquired resistance (*vide infra*) Thus infections with some strains of *Bor recurrentis* are readily cured by salvarsan drugs, while others do not respond In the case of *T brucei* in mice, it has been found that a strain when recently passed to this species of host was less sensitive to chemotherapeutic agents than after repeated passages, when it had increased in virulence (Browning and Gulbransen)

Acquired drug-resistance ('drug-fastness') was discovered in the case of *Trypanosoma brucei* infection in mice by Franke and Roehl A drug fast strain may be produced as follows Animals are inoculated and then treated with a dose of the drug which does not lead to complete sterilisation of the infection, although the parasites disappear from the blood for a time, a relapse occurs The relapse may again be susceptible to treatment with the same drug, but another relapse follows After repeated relapses treated in this way there may finally be no response to the largest dose of the drug Now, when the parasites are inoculated into a fresh animal the infection which develops also fails to be influenced by the drug Such drug-fastness may persist unaltered when the infection is afterwards passed for long periods through untreated animals Thus, some change has occurred in the parasites themselves As will be seen later, the parasites from a relapse are serologically different from those which constitute the original infection, drug-fastness without serological change in the trypanosomes may be produced as follows

Animals are inoculated and then treated with a dose of the drug too small to influence the course of the infection From these the trypanosomes are passed to fresh animals which are treated similarly It is found that as the number of passages progresses, the dose of the drug which may be given without obviously influencing the multiplication of the trypanosomes, increases until the largest tolerated dose is without therapeutic effect Thus finally a large multiple of the original curative dose of a highly effective drug may fail to delay the course of the infection Another procedure consists in subjecting the organisms to contact with the drug *in vitro* between successive animal passages Clinically the conditions conducive to the production of drug fast organisms may be created whenever treatment short of producing complete sterilisation has been carried out over a period, or, as has been seen above, where a series of relapses has been treated with the same drug Some compounds are more prone to produce drug-resistant parasites than others Again, while in some cases resistance may be shown only towards the compound which developed it and to others chemically closely related, on the other hand resistance may also be manifested towards drugs of widely different chemical constitution It must be emphasised however, that the

development of drug-resistance is not the inevitable result of such procedures. The parasites may be destroyed ultimately after repeated relapses, each treated by the same dose of the drug as failed to sterilise the infection originally (Browning and Gulbrandsen; Calver). In the malaria group also, drug-resistance has been found; thus *P. knowlesi* in monkeys treated with pamaquin readily develops resistance, which is fairly stable (Fulton and Yorke). Similarly, *P. gallinaceum* acquires resistance to paludrine (see Williamson and Lourie).

The question of maintenance of virulence requires to be considered in relation to drug-fastness, since the danger has to be considered of producing organisms which, while highly pathogenic, are no longer capable of being influenced by chemotherapeutic agents. In the examples quoted drug-fastness has been acquired by parasites which retained their full virulence. By subjecting cultures of bacteria to increasing concentrations of an antiseptic *in vitro* it is possible to produce resistant strains; however, in the process the organisms frequently lose their original virulence. On the other hand, fully virulent strains of pneumococci resistant to the sulphonamide drugs have been developed and also meningococci resistant to streptomycin.

It remains undecided whether drug-fastness may be acquired by parasites which originally were sensitive or whether it merely represents a natural variation undergone by a few individuals in a population of organisms. In the latter case, the resistant 'mutants' which at first were present in very small proportions, will become predominant when the sensitive organisms are eliminated by the therapeutic agent (see Alexander and Leidy).

Serological Resistance (Serum-fast or Relapse Strains)

Ehrlich and his associates first discovered in the case of *T. brucei* that when infected mice were treated so that the parasites disappeared temporarily from their blood, but a relapse occurred, these relapse parasites were serologically different from the original strain. This was demonstrated by infecting fresh mice with the relapse parasites and producing sterilisation by any effective drug. The cured animals were then reinoculated, some with the original strain and some with the relapse strain. There was immunity to the latter (as shown by development of the infection only after a prolonged incubation period, this protective power depending upon antibodies in the serum), but not to the former. Similarly, animals infected with the original strain and then cured were immune to it, but not to the relapse strain. In the same way, when mice which had been infected with a strain of trypanosomes and cured were reinoculated with the same strain, the parasites which afterwards appeared in the blood had the characters of a relapse strain. A relapse strain may also be produced when the original strain is brought into contact for a short time *in vitro* with the serum of an animal which has been infected with it and then cured, provided that the serum is insufficient to kill the parasites; the treated trypanosomes are washed free from the serum and used to infect a normal mouse. By repeated treatments short of complete cure, parasites from a series of successive relapses may be obtained. These are found to consist of a considerable number of serologically distinct strains. Similar results are observed in the case of animals such as rabbits, in which the infection spontaneously pursues a relapsing course. Thus when a relapse occurs the parasites acquire different antigens. In the case of a

strain of *T. congolense* which without treatment pursues a relapsing course in mice, the events are different—while the relapse parasites acquire new antigens, they do not lose the old ones. Accordingly, an animal which has been cured of infection with a relapse strain and has thereby acquired immunity to this, is also immune to the original strain—but of course the converse does not hold (Calver). So far as is known, the serological modifications characteristic of relapse strains are confined to trypanosomes and to spirochetes of the relapsing fever group.

Chemotherapeutic Potentiation and Interference

Two chemotherapeutic agents, which damage the parasites through different mechanisms, might be expected to assist the therapeutic action of each other, so that administration together of less than half the curative dose of each, would bring about cure. There is evidence that sometimes this occurs; but the examples of potentiation which have been discovered so far are not on a marked scale. On the other hand, chemotherapeutic interference has been very strikingly exemplified in the case both of trypanosomes and bacteria. It was originally shown by Browning and Gulbransen with *T. brucei* that a chemotherapeutic drug when given in a non-curative dose might prevent the action of one of different chemical constitution administered shortly afterwards in a dose which otherwise would have influenced the infection. The likelihood of any direct physical or chemical action between the two compounds was practically excluded. The phenomenon is not connected with acquired drug-resistance. Selbie, basing his experiments on Woods' observations *in vitro*, then demonstrated a similar interference phenomenon in mice inoculated with virulent streptococci and treated with sulphonamide drugs, the administration of *p*-amino benzoic acid to the animals preventing therapeutic action. Other examples of interference have been recorded. The effect of the 'antagoniser', as it has been termed, has been attributed to its being normally an essential metabolite for the organism, which the chemotherapeutic agent tends to render unavailable by combining with the same enzyme mechanism (Hildes). This is supposed to occur in virtue of structural similarity in the chemical sense between the metabolite (antagoniser) and the drug. Thus the chemotherapeutic agent, by blocking, the assimilation of a foodstuff required by the bacterium, would lead, as it were, to starvation of the latter. Strains rendered drug fast by growth in the presence of sulphonamides have been stated to contain as much as seventy times the amount of *p*-amino benzoic acid present in the susceptible strain. This explanation obviously does not apply in the case of chemotherapeutic interference of trypanocidal agents described above. It is probable that interference effects depend on various mechanisms.

The Role of the Natural Defences and of Acquired Immunity in Chemotherapeutic Action

It has been concluded that the curative effect of chemotherapeutic agents frequently depends on the co-operation of the natural defences, as well as processes as phagocytosis of the parasites are interfered with for the purpose of the drug. Much convincing evidence is afforded by the observation that a mixture of virulent organisms, e.g. streptococci, with a chemotherapeutic agent, may be fully responsible for an interdependent mechanism, although the concentration of the compound used does not prevent the death of the organism in mice till after phagocytosis has taken place. A similar phenomenon

administered before inoculation of mice with trypanosomes may not prevent the latter from multiplying initially and appearing in considerable numbers in the blood, and yet this may be followed by their disappearance and permanent cure of the infection. Similarly, under treatment with drugs of the sulphonamide group, mice infected with highly virulent streptococci may show a prolonged, symptomless infection, the organisms in the blood becoming non-virulent for fresh animals, and the infection finally being sterilised after several weeks, presumably by the tissue defences (Browning and Leckie). Of course, where the organisms reach considerable numbers in the body, conditions are favourable for the development of immunity. In infections, such as that with *T. congolense* in mice, cure by drugs is less readily effected at an early stage when parasites are scanty than later when they have become abundant. This points to the part played by the immunity reaction in bringing about sterilisation. On the other hand, with trypanosomes of the *T. brucei* group the opposite holds, cure occurring more readily at an early stage of the disease than later. But with these parasites also, immunity reactions may play an important part in effecting cure, as was shown by Schnitzer and Silberstein. They inoculated mice with a strain of *T. brucei* susceptible to an arsenical drug, and this was followed one or two days later by inoculation with a derived strain which had been rendered resistant to the arsenical, but was serologically identical with the original strain. When parasites were abundant in the blood the arsenical compound was administered in a dose sufficient to sterilise the infection with the sensitive trypanosomes, as a result complete sterilisation was effected. This is scarcely explicable except on the basis of an immunity reaction to the trypanosomes of the sensitive strain destroyed under the influence of the arsenical, the destruction of the drug-fast trypanosomes then being brought about by the antibodies so produced. *Tr. pallidum* is an outstanding example of an organism which causes infection that is more readily influenced at the early stage. It would appear that the drug enters the host

The Mode of Action of Chemotherapeutic Agents

It is obvious that an effective chemotherapeutic agent in the dosage employed must have a selective action in damaging the metabolism of the parasites without seriously poisoning the tissues of the host. Ehrlich summed up the properties which such drugs should possess in the statement that chemotherapeutic action depends on the drug being fixed by chemical structures of the susceptible parasites, which he called 'receptors', and that the affinity of the drug for these receptors (parasitotropism) must be greater than its affinity for the tissues of the host (organotropism). Accordingly, chemotherapeutic agents differ in their behaviour from substances such as phenol, which are protoplasm poisons inimical to all forms of life. Little is known regarding the nature of the action mechanism by which chemotherapeutic agents damage the parasites, however. Thus the finding that a drug interferes with one or other of the enzyme activities of the parasites does not necessarily mean that this is the essential mode of its action on the parasites. For example, a trypanocidal drug such as *arsenic* is sensitive to the action of the host's tissues, but is not absorbed by the strain which it is intended to destroy. But this does not prove that the dye must enter into the interior of the trypanosomes before it can injure them. It may well be that the damage takes place at the surface and that permeability of the protoplasm to the dye

is a result. In the case of arsenical trypanocidal agents it is possible that the essential mechanism is the affinity of the arsenical for sulphhydryl groups in the trypanosomes; in other words, as suggested by Elulich and Voegtlin *et al.*, the -SH group constitutes the receptor. King and Strangeways have shown that the primary action on the parasites produced by these arsenical drugs may take place in three different ways according to their chemical constitution, *viz.* (1) by ions acting in a watery medium, *e.g.* the neutral sodium salts of carboxyphenyl arsenoxides, (2) by compounds without hydrophile groups apart from the arsenoxide group, *e.g.* phenyl arsenoxide, which are probably taken up at a lipid-water interface with the phenyl group in the lipid and the arsenoxide group in the water—since a large proportion of the trypanosome body consists of lipoids, conditions are favourable for this means of transport of the drug within the parasites, (3) by those compounds that are fixed by the same type of structures in the trypanosome cell as fix trypanocidal dyes of the acridine and oxazine groups, *e.g.* *p*-acetanilide arsenoxide. The second group is the most actively lethal for the trypanosomes *in vitro* and the first is the least potent. Strains of trypanosomes which have been rendered drug-fast to compounds of the third group, are not more resistant than the normal strain to members of the other two groups, a finding which is in favour of the mode of action of the third group being distinct.

As regards the mode of action of penicillin on Gram-positive cocci recent work of Gale and Taylor suggests that the drug affects the cell wall of the organisms during growth in such a way that assimilation of glutamic acid is prevented, and possibly other nutrients for the organisms may be similarly interfered with. On the other hand, sulphathiazole, while preventing the entrance of glutamic acid into the bacterial cell, interferes with its condensation into peptide (protein) form in growing cells. It has already been mentioned that the action of sulphonamide drugs on bacteria is antagonised by *p*-amino benzoic acid (although there are exceptions, *e.g.* *p*-amino methyl benzyl sulphonamide or marfanil). At the same time it has been shown that this acid is an essential metabolite for certain bacteria. Accordingly, the view has been expressed that in searching for chemotherapeutic agents attention should be directed especially to compounds which have a chemical constitution resembling food stuffs of the organisms (see *supra*). On the basis of the antagonism between pantoic acid and sulphonamides, the growth-promoting effect of salts of pantothenic acid for streptococci, Mellswain and Hawking predicted the chemotherapeutic action of taurine in streptococcal infections. This was verified for rat bacillary dysentery, mice, the pantothenic acid content of the tissues of the fatality group being prevented the therapeutic effect. However, the possibility of using taurine as a guide to preparing new effective chemotherapeutic agents is doubtful.

Certain compounds which are chemotherapeutic *in vitro* have only at most only a weak or slow lethal action on the organisms *in vivo*, while at the same time there is no evidence that they change into more active derivatives in the body of the host. Examples are suramin (Bayer-205) and *in vivo* cases is that in the presence of the compound the infecting organisms is unable to progress, continuous treatment eventually destroyed by the natural defences of the host. The chemotherapeutic agents so far known are of exotoxins. Accordingly, when the infecting organism is destroyed, the combined administration of antitoxins and antibiotics is essential.

antitoxic serum may be expected to prove more effective than the drug alone. This is well shown with penicillin or drugs of the sulphonamide group in experimental staphylococcus infection (P. Browning and Calver).

CHEMOTHERAPY IN RELATION TO PARTICULAR INFECTIVE AGENTS

Trypanosomes

Owing to their relatively large size and structural differentiation trypanosomes lend themselves specially to detailed study, and they were the first infective agents to yield highly successful results. There are now drugs belonging to a considerable number of chemical classes which can influence or cure trypanosomiasis, especially in small animals experimentally inoculated. The following are among those effective against the *T. brucei* group—arsenicals, especially certain trivalent aromatic organic derivatives (salvarsan, arsenophenylglycine, mapharside); pentavalent organic arsenicals (atovyl, tryparsamide), compounds of antimony and of bismuth, bisazo dyes (trypan-red, trypan-blue), suramin (also called Bayer-205, germanin, etc.—related to the previous group, but not a dye); the diamino-acridinium dye trypanflavin (acriflavine); styryl quinoline dyes (styryl-314); triphenyl-methane dyes (parafuchsine, trypanosan); diamidine compounds (stilbamidine, etc.). Owing to its slow excretion from the body suramin has a prophylactic action extending over many weeks. In man infection with *T. rhodesiense*, especially in the early stage, responds well to suramin, while *T. gambiense* is also susceptible to arsenicals, e.g. salvarsan or tryparsamide. When the central nervous system has become infected tryparsamide is of greatest use owing to its passing into the cerebro-spinal fluid. Suramin has proved valuable, too, in various animal trypanosomiasis, e.g. in camels in the Sudan. Potentiation with drugs of two different chemical types, as well as chemotherapeutic interference, are met with in the case of the *T. brucei* group. *T. congolense* infections respond to most of the above drugs, but are more resistant to the high dosage, 1 g. 4 times a day.

Suramin is also effective against *T. brucei* in cattle. *T. cruzi* so far responds to none of the above drugs, but is highly susceptible to the diamidine compounds, e.g. stilbamidine bromide, by Bayer-70. Diamine drugs (e.g. 3-carbethoxyamino 9-*p*-carbethoxyamino 1-methyl phenanthridinium chloride), which do not act on *T. brucei* or *T. congolense* except in the highest doses.

Acquired drug-resistance tends to be produced by prolonged non-sterilising treatment in the case of trypanosomes of the *T. brucei* group. On the other hand, it appears to be difficult to render *T. congolense* drug-fast in mice. Acquired drug-resistance is retained by the trypanosomes after cyclical development in the insect host.

Spirochaetes

Tr. pallidum. The antisyphilitic property of mercurials is relatively weak and bismuth has largely replaced that metal. Ehrlich and Hata, proceeding on the analogy of the action on *Sp. minus* in mice, discovered the powerful therapeutic property of the trivalent organic arsenical salvarsan (and its analogues) in experimental *Tr. pallidum* infection in rabbits, and subsequently its use in man revolutionised treatment of the disease. The

corresponding arsenoxide (mapharside) is also highly effective. Penicillin has a powerful and rapid action on infection with *Tr. pallidum*. The subject of antisyphilitic treatment is one of great complexity and cannot be gone into here. Bismuth, arsenicals, and penicillin are effective in yaws. The syphilis spirochete does not seem to acquire a high degree of drug-resistance.

Relapsing fever spirochetes Although salvarsan is highly effective with many strains of the relapsing fever spirochete, certain are resistant. Penicillin acts on *Bor. recurrentis* infection (Lounie and Collier).

Spirochaetosis of birds is very susceptible to arsenicals.

Sp. minus infection (rat-bite fever) affords a striking example of a condition which is more susceptible to the salvarsan drugs in man than in small animals. This organism is also sensitive to penicillin.

Leptospira infections are not readily influenced by any of the known antispirochetal drugs except penicillin (see Larson *et al.*, Petersen *et al.*).

Malaria Parasites

Quinine and certain of its analogues, as well as methylene blue and the salvarsan group, all more or less effectively 'suppress' malarial infections since continued administration leads to reduction in the number of parasites in the blood and disappearance of symptoms. In addition new highly effective antimalarials have been discovered as a result of experimental investigations chiefly on analogous infections in birds—pamaquin (or plasmoquin—a quinoline derivative), mepacrine (or atebrin—an acridine derivative) and paludrine (a phenyl biguanide compound). Undecane diamidine also has therapeutic action on *P. vivax* infections. It must be remembered that a drug does not necessarily affect in parallel fashion the different animal and human malarial parasites (see Curd). Thus sulphonamide compounds act on various animal malarias, e.g. *Rhesus* monkeys are readily cured of the very virulent infection with *P. knowlesi*, and *P. falciparum* is influenced, but there is only a relatively weak effect on the other human malarias or on the mild infection with *P. mous* in monkeys. Mepacrine, like quinine, acts on all the human parasites in the phases of their asexual cycle, thus it is very effective in suppressing the signs of infection in man. It also acts on the benign tertian and quartan gametocytes, but not on the crescents. Pamaquin on the other hand is active against the crescents of *P. falciparum* and the gametocytes of *P. vivax* and *P. malariae*, and it has a moderate effect on the asexual cycle of the two latter, but not on that of *P. falciparum*. The damage inflicted by this drug on the crescents prevents transmission of *P. falciparum* infection to the mosquito. Neither quinine nor mepacrine has a prophylactic action when administered shortly before and for a few days after the bite of the infected mosquito, similarly neither drug influences the exoerythrocytic stages of the parasites as determined in experimental infections with certain species of bird malaria. Accordingly, although infection is suppressed, they do not act as 'causal prophylactics'. Pamaquin has this prophylactic property and it is very pronounced in the case of paludrine which destroys the exoerythrocytic forms and so may be regarded as a true causal prophylactic. No compound so far discovered destroys the sporozoites. The above results with the various drugs have been confirmed for human malaria in volunteers (Fairley, p. 656). Probably also none effects complete sterilisation of *P. vivax*, when administration is discontinued an attack of malaria develops after a latent period which may extend to months. Finally, sterilisation seems to be brought about by the tissue defences. In the treatment of malaria it is important to estimate the

concentration of the drug in the patient's blood as a guide to dosage, e.g. of mepacrine, owing to the individual variations in absorption and other factors on which the blood level depends (see Reid). There is experimental evidence that drug-resistance to pamaquin and paludrine may be developed by malarial Plasmodia.

Other Protozoa

Leishmaniae. The visceral type of disease responds well to chemotherapeutic agents, but the muco-cutaneous form is refractory. Both antimonials, especially pentavalent organic compounds, since they are less toxic than trivalent antimony, and the diamidines (stilbamidine and pentamidine of Lourie and Yorke) act on leishmaniae. Goodwin has shown that in inoculated Syrian hamsters (*Cricetus auratus*), by measuring the ratio of the number of parasites to the number of nuclei in imprint preparations from the freshly cut surface of the spleen a fair estimate of the degree of infection can be obtained. Thus a reliable method is provided for comparing the therapeutic action of different drugs.

Piroplasms. Nuttall and Hadyen originally pointed out the chemotherapeutic action of trypan-blue on *Babesia canis*. Acriflavine also acts on some babesia infections. Certain of the amidines (propamidine, pentamidine) are highly effective against diseases caused by babesia in various domestic animals (Lourie and Yorke), but not against *Theileria annulata*, *Anaplasma marginale* or *Anaplasma ovis* (Adler *et al.*). Non-sterilising treatment with stilbamidine readily leads to drug-resistance of *Babesia canis* in several passages, and the resistance persists when the parasites are afterwards passed through a long series of untreated animals (Fulton and Yorke). The parasites are found to be resistant also to acaprin, which is chemically of quite different constitution. The latter compound acts on some babesias and on certain of the minute theileria infections, e.g. *Theileria annulata*, which do not respond to the other drugs.

Entamoeba histolytica. Emetine, which is powerfully lethal for *E. histolytica* in cultures *in vitro*, acts also *in vivo*, but is not highly effective clinically. By the use of young rats experimentally inoculated with the amoebae intracacally a procedure has been evolved which enables different therapeutic compounds to be compared (Jones). Of the substances examined the most active were chiniofon (yatren or loretin), mepacrine and carbarsone. Emetine hydrochloride was not highly effective; it appears, however, that different strains of *E. histolytica* vary considerably in their therapeutic response to this drug.

Bacteria

A practical distinction must be made between infections which can only be reached by compounds circulating in the blood or lymph and infections at readily accessible sites. The latter may be influenced by the application of 'surface antiseptics', as well as by drugs which are conveyed through the circulation. Organisms contained in necrotic tissues or collections of pus are likely to be inaccessible by any means. It is not yet clear in how far there is the danger of producing drug-resistant strains of virulent bacteria by prolonged treatment which does not lead to sterilisation. Experimentally, virulent pneumococci have been obtained with acquired resistance to sulphonamide drugs.

Both general and local infections with streptococci and penicillin. Both general and local infections with streptococci and penicillin. Both general and local infections with streptococci and penicillin.



Tubercle bacillus. Sulphone compounds (diamino diphenyl sulphone, promin, etc.), which are closely related chemically to the sulphonamide group, and also the antibiotic, streptomycin, have a marked therapeutic effect in guinea-pigs infected with tubercle bacilli of human type (see Feldman). When treatment was begun some weeks after inoculation and continued for seven months to a year, the majority of animals were alive and appeared well, while in this time 80 per cent. of the untreated controls died. In the treated animals visceral lesions retrogressed; however, the infection, though suppressed, was not sterilised. Also there was no acquired immunity in this very susceptible species, since on the cessation of treatment the disease again progressed. Successful results have also been obtained occasionally with gold compounds. Streptomycin has proved of value in human tuberculosis for the treatment of early acute infections, especially military tuberculosis and tuberculous meningitis. About 50 per cent. of such treated cases have shown remissions, which have been observed for upwards of 2 years. There seems to be a considerable risk, however, that under continued administration a drug-fast infection may replace that which originally was sensitive (Feldman and Hinshaw).

Actinomyces and pathogenic fungi (Sporothrix, Blastomycetes) respond in some cases to large doses of iodides. Good results have also been reported in actinomycosis from the use of penicillin or sulphonamide drugs.

Bartonella The condition in splenectomised rats can be readily prevented by salvarsan drugs. But these do not appear to act in the human disease.

Rickettsiæ

Certain sulphonamide compounds, especially *p*-sulphonamido benzamide hydrochloride and the corresponding amidoxime and close analogues, prepared by King and Walker, when injected intraperitoneally or subcutaneously have a definite effect in preventing the development of the characteristic lung lesions in mice inoculated intranasally with suspensions of typhus rickettsiæ, either of murine or epidemic type (Andrewes, Begg and van den Ende). Protection is also conferred against intraperitoneal infection with murine typhus rickettsia, but not against the toxic effects. Guinea-pigs are not protected against the febrile reaction produced by intraperitoneal inoculation with epidemic typhus rickettsia, and when administered to florid human cases the disease is not favourably influenced. Toluidine blue and methylene blue *per os* and related dyes have some therapeutic effect in mice and *p*-amino benzoic acid acts slightly. Penicillin also is active.

Viruses

The virus of lymphogranuloma inguinale responds to treatment with drugs of the sulphonamide group both in the human subject and also in experimental infection of animals. It has been reported that compounds of antimony also have a therapeutic effect. Viruses of the psittacosis group are influenced by sulphonamide drugs and by penicillin. Apparently no other viruses are susceptible to known chemotherapeutic agents (see Kramer *et al*, Cutting *et al*).

CHAPTER XXXVIII

THE BACTERIAL FLORA OF THE NORMAL SKIN AND MUCOUS MEMBRANES

THE skin and mucous membranes, in virtue of being in more or less direct contact with the outer world, are liable to contamination with various micro-organisms. In addition, the local chemical and physical conditions at a surface may determine what organisms will multiply there; also, the normal flora may influence the growth of any superadded bacteria. As will be seen, the commensal flora in different parts of the body may be very diverse. It is obvious that when considering what constitutes an abnormal flora, which may have pathogenic significance, it is necessary to know the normal condition both in its qualitative and quantitative aspects. Accordingly, the organisms should be studied both in direct films and also in cultures on various media. Types which are abundant may fail to grow on ordinary media, e.g. spirochaetes, or as in the case of *B. bifidus*, the predominating organism in the faeces of breast-fed infants, may require anaerobic conditions for their initial growth. The use of selective enrichment media may be necessary in order to demonstrate the presence of organisms which are either extremely scanty, such as *B. aerogenes* in normal faeces, or which are readily overgrown when along with the usual coliform bacilli. Pathogenic forms may be transiently present without disease resulting. The conditions which determine the normal flora of a part are as yet only imperfectly understood. Accordingly one can only note the fact that in different parts of the world the organisms found, say in the upper respiratory tract, may vary.

The Skin. The exposed skin surface, except at flexures, is too dry to permit free growth of bacteria, also a self-sterilising action has been attributed to it, whereby most bacteria placed on the surface disappear. But the nature of this action has not been defined, it is not merely a function of the natural acidity of the secretions and little is known about its variations under different conditions. On the other hand, certain organisms seem to have a natural habitat on the skin surface, in the intercellular spaces of the epidermis, and in the sweat and sebaceous glands. It is therefore necessary to distinguish between a 'transient' and 'resident' flora of the skin. The latter is exemplified by the staphylococci (*Staphylococcus albus* most frequently) and certain diphtheroid bacilli, etc. It is significant that *Staphylococcus aureus* is habitually present on the skin of the face and neck and that this 'skin-carnage' is commonly maintained by its presence in the nose. Where surface contamination is abundant, as at muco-cutaneous junctions, the organisms from the mucous membrane are numerous, e.g. coliform bacilli, sporing anaerobes such as *B. Welchii*, and the enterococcus, near the anus. The preputial secretions favour the growth of the smegma bacillus, Gram negative and -positive cocci, diphtheroid bacilli, *B. fusiformis*, and various spirochaetes. The external genitalia of the female have a similar flora. Where there are fatty and waxy secretions (as in the ear, genitalia, and pilo-nidal cysts) acid-fast organisms such as the smegma bacillus tend to flourish. On the conjunctiva *Staphylococcus albus* and diphtheroid bacilli, such as *B. xerosis*, are common. Various bacteria and fungi present in air and dust may be found on the skin, and in addition to the species mentioned above a great

variety of organisms may be isolated in cultures from the skin, e.g. *Micrococcus tetragenus*, *sarcinæ*, *B. proteus*, *B. pyocyaneus*, and various moulds and yeasts. Streptococci both of hæmolytic and non-hæmolytic classes may also be found apart from any definite pathological condition. Occupational and other factors may influence the skin flora (Lominski and Thomson). It should be emphasised that complete sterilisation of the skin can seldom be effected by the application of antiseptics, and in some persons *Staphylococcus albus* or *aureus* and other organisms may grow deeply, only coming to the surface as a result of continued sweating.

The Mouth. In the mouth a great variety of organisms is found and any accumulation of secretions or epithelial debris leads to increase in their number. Streptococci of α and γ types, spirilla and vibrios, leptothrix and streptothrix forms and also potentially pathogenic types such as the pneumococcus, staphylococci, and fusiform bacilli are all frequent. In the interstices between the teeth and on the gums, abundant spirochaetes of various kinds are often present. Where there are carious teeth, aciduric organisms such as *B. acidophilus odontolyticus* are present, but it is uncertain in how far this is the cause of caries.

The Respiratory Tract. While the nasal vestibule contains many and varied organisms, *Staphylococcus albus* and *aureus*, diphtheroids, etc., the nasal fossæ, so far as they are accessible, are practically sterile. Even slightly abnormal conditions, however, such as those associated with the common cold, lead to the appearance of organisms, which may be numerous, such as staphylococci, streptococci, Friedlander's bacillus, *D. catarrhalis* and diphtheroid bacilli. These probably prolong the effects of the original infection, although they may not initiate it. The nasal mucous membrane has a marked capacity for ridding itself of bacteria placed on it; the mechanism involved is not clear, however, although it seems not to depend entirely on mechanical removal. The naso-pharynx harbours non-hæmolytic streptococci, non-pathogenic Gram-negative cocci, pneumococci (chiefly of Group IV), staphylococci, bacilli of the influenza group, and minute Gram-negative, non-sporing anaerobic bacteria exemplified by *Bact. pneumosintes*. The paranasal sinuses are normally sterile. Friedlander's bacillus is a common inhabitant of the upper respiratory tract and occurs also in the mouth. The trachea, bronchi, and pulmonary tissues in the healthy state are practically free from organisms. It is difficult to define sharply the limits between the normal flora mentioned above and the transient presence of potential pathogens. Thus hæmolytic streptococci, type III pneumococcus, Pfeiffer's bacillus and the meningococcus are frequently isolated from naso-pharyngeal swabs taken from an apparently healthy section of an urban population. The distribution of organisms tends to vary with the season, there being an extra-nasal cavity and downwards in the trachea and larger bronchi during the cold period of late winter and early spring.

The Alimentary Tract. In the alimentary canal organisms are swallowed with the food and saliva and are destroyed by the acid of the gastric juice, so that when the contents are nearly sterile. The flora of the small intestine, which are of the intestinal type, is abundant after the first 2 to 6 ft. The flora is abundant in the ileum and reaches its maximum in the large intestine. Lower down the large intestine, the death of a considerable proportion of the flora occurs. The third of the dry weight of the faeces consists of the different types dependent on the diet.

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the diet, but the principal forms are coliform bacilli, *B. proteus*, enterococcus, bacilli (*B. welchii*, *mesentericus*, etc.)

The organisms present in the intestines may be classified according as they are saccharolytic (enterococcus, *B. acidophilus*, *B. welchii*) or proteolytic, i.e. 'putrefactive' (*B. sporogenes*, *B. proteus*, *B. pyocyaneus*, *B. mesentericus*). Diet is believed to influence the flora, a large proportion of meat conducing to the proteolytic types, whereas carbohydrates—especially lactose which is supposed to reach the lower parts of the intestine before being split up—lead to the preponderance of an aciduric flora. Feeding with soured milk assists in establishing the latter. It is not easy, however, to cause an organism foreign to the individual's intestine to flourish there, although abnormal conditions,

species, including specific pathogens. It is doubtful whether bacteriophage plays much part in regulating the intestinal flora. Bacteria which have been swallowed appear in the dejecta of new-born infants within twenty-four hours, at first they are of various kinds but, when breast-fed, *B. bifidus* soon becomes

infants where the alimentary tract is normal, the upper part of the small intestine is nearly sterile, and that this is not due to the acidity of the contents or to the presence of bacteriophage.

The Female Genital Tract. In the adult female the vaginal secretion is typically acid from lactic acid derived from glycogen secreted in the lining epithelium. This state persists from the onset of puberty until the menopause. The acid secretion is unfavourable for the majority of organisms and the characteristic flora consists of the aciduric Döderlein's bacillus (*B. acidophilus*). In a proportion of pregnant women, however, the flora consists to a greater or less extent of other organisms such as streptococci, coliform bacilli, diphtheroids, yeasts, and sarcinae. In children from three to four weeks old until puberty, the vaginal secretion is alkaline and contains few organisms, chiefly cocci.

CHAPTER XXXIX

THE BACTERIOLOGY OF AIR, WATER, SEWAGE, SOIL, AND MILK, FROM THE HYGIENIC STANDPOINT

AIR

THE atmosphere is not a natural habitat of micro-organisms, though fungi and bacteria are constantly present in it. Such organisms are introduced into the air from various substrates in which they flourish. Thus, particles of air-borne dust from the soil, streets, dwellings, etc., dried material shed from the bodies of animals and man, dust from human clothing, bedding, etc., and droplets expelled from the respiratory passages create the apparent micro-flora of the atmosphere. Any factors which increase the amount of suspended matter in the air add to the bacterial content. Therefore bacteria are most numerous in the air of inhabited places, particularly within enclosed, badly ventilated, and crowded premises. On the other hand, at high mountain altitudes and over deserts the air contains relatively few bacteria, while over the sea the number increases with proximity to the shore, but not even the stratosphere is bacteriologically sterile (W. F. Wells and M. W. Wells; Proctor). Bacteria when introduced into the air remain there for only a limited time, as dust particles and droplets settle by gravity; the contained organisms are removed from the atmosphere and the rate of removal depends on the size of the particles, the larger settling out rapidly, the smaller remaining suspended for a time and also diffusing for some distance.

The micro-flora of the air consists mainly of the spores of various fungi, spores of saprophytic bacteria (*B. subtilis*, etc.), and a variety of micrococci, e.g. saprophytic staphylococci and sarcinae. Some of these are specially adapted for air distribution. Thus, the spores of fungi and bacteria can resist natural desiccation in air and dust provided they are not exposed to direct sunlight. Air samples regularly contain these organisms, which are common inhabitants of soil and decomposing organic material. But many non-sporing bacteria can also survive in desiccated material and are found in air and dust. The pathogenic bacteria vary in this respect, some being susceptible, others resistant to natural drying, and thus is a determining factor in the spread of various infections by air and dust. Among the pathogenic organisms which may readily gain access to air and can survive in the dry state for a considerable time the following may be included: the tubercle bacillus, staphylococci, streptococci, pneumococcus, diphtheria bacillus, spores of the anthrax bacillus (as in wool factories), spores of anaerobic bacilli (*B. tetani*, *B. welchii*, etc.) and the spores of certain pathogenic fungi. Many of the viruses also have considerable resistance to drying, e.g. of smallpox, vaccinia, psittacosis, poliomyelitis, influenza (see Landsteiner and Levaditi; Edwards). The actual survival in the dust of infected premises has been fully established in the case of various pathogenic agents, e.g. pyogenic staphylococci and streptococci, pneumococcus, diphtheria bacillus, tubercle bacillus, and the poliomyelitis virus (see Kirstein, Cruickshank, White; Brown and Allison, Avery *et al*, Hare; Wright *et al*; Neustadter and Thro). The stirring of infected dust is undoubtedly an important means whereby the atmosphere becomes contaminated with micro-organisms.

Flügge in early studies of air-borne infection pointed out that secretion droplets from the respiratory passages may float in the air for several hours and are transported by air currents, and he regarded the diffusion of suspended droplets as an important process whereby certain pathogenic organisms are spread from one person to another. The later work of Wells has added to our knowledge of this subject. He showed that droplets of 0.1 mm. diameter or more settle rapidly while smaller droplets tend to remain suspended. The latter, however, evaporate giving rise to 'droplet-nuclei' of dried material, these float for a considerable time in the atmosphere and are carried some distance by air currents. If pathogenic organisms should be present and remain viable in droplet-nuclei, the potential spread would be considerable. Among the variables that might determine air distribution of bacteria is the vigour of expulsion of droplets from the respiratory passages, and sneezing is the most effective in this respect. Bourdillon and his co-workers showed that an average sneeze leads to the emission of 100,000 bacteria-containing particles of a size small enough to remain in the air of a closed room for more than one minute and that 4 per cent. of these remain suspended for thirty minutes. The diffusion of bacteria when contaminated droplets are introduced into the atmosphere has been fully demonstrated by various observers.

to the possibility of air-borne infection by means of suspended droplet-nuclei, but, in general, contamination of the air in this way by pathogenic organisms would be relatively dilute unless under conditions of overcrowding and bad ventilation, for the larger droplets containing most bacteria from the respiratory passages settle rapidly, and contaminate the dust of premises, clothing, etc., rather than the air itself. Recent work has on the whole tended to minimise the importance of droplet infection and to emphasise the part played by dust in the spread of pathogenic organisms in the air. According to Hare, haemolytic streptococci are expelled from the mouth and throat mainly in the larger droplets which at once settle, and his findings support the view that the danger of direct air-borne infection is comparatively slight and that the contamination of clothing, bedding, etc., with subsequent stirring up of infected dust particles from these is the usual mode of spread of respiratory tract infection (see Hare and Mackenzie).

The question arises as to the degree of infectivity of the smaller droplets which are likely to remain suspended in the air. This has recently been studied by Duguid who points out that the great majority of droplets originate from the front of the mouth and that their infectivity will depend on the presence and number of pathogenic organisms in this region. He found haemolytic streptococci in the anterior-mouth secretions in only 13 of 87 patients with throat infection due to these organisms, during a series of six coughs infected droplets were expelled by 39 of these. He obtained analogous results in persons with diphtheria infection of the throat and in cases of pulmonary phthisis. He has also shown in cases of respiratory tract infections that only few of the smaller droplets which become floating droplet nuclei are likely to contain pathogenic bacteria. While this may be true for bacterial diseases, in virus infections of the respiratory tract the concentration of the infective agent in the secretions of the mouth may be much greater and in such cases droplet nuclei may be more likely to spread the infection.

On the whole present evidence points definitely to the greater likelihood of air-borne infection being due to the stirring of infected dust from premises.

clothing, bedding, etc., than to infected droplet-nuclei. Of course the possibility must be remembered that recently expelled and suspended droplets from an infected person may enter the respiratory passages of another in the immediate vicinity or contaminate the skin or clothing of such person, but this is not air-borne infection in the strict sense, and organisms with only feeble viability outside the body, e.g. the meningococcus, may be conveyed in this way, apart from those which are capable of resisting natural desiccation.

Method of Plate Count. The simplest procedure is to e... s of culture medium. The organisms present in dust particles or droplets settling on the medium form colonies on incubation and so a qualitative and quantitative examination can be made. This method has proved useful for detecting such organisms as hæmolytic streptococci, blood agar being used as the culture medium. Of course, colony counts as indicators of the degree of air contamination can only be of comparative value and the method is not an efficient means of detecting organisms in the smallest suspended particles, such as droplet-nuclei.

More elaborate methods have been devised for bacteriological sampling of air with the particular object of detecting organisms in the finest particles. Highly satisfactory results have been obtained with the 'sbt-sampler' devised by Bourdillon and his associates (1941). By this instrument a given volume of air is directed through a slit 0.25 mm. wide, on to a rotating plate of culture medium just under it. The rate of passage of the air through the slit is adjusted to 1 c.ft. per minute and samples up to 10 c.ft. are examined. The rotation of the plate ensures even distribution of the organisms. For detecting streptococci it is advantageous to use blood agar containing 1 : 1,000,000 crystal violet, which inhibits most of the ordinary air and dust organisms while allowing both hæmolytic and viridans streptococci to grow. The latter organism serves as an indicator of naso-pharyngeal contamination of the air. The 'air centrifuge' devised by Wells and his associates (1937) is also suitable for the detection and enumeration of bacteria present in the finest particles.

Air Disinfection. Much work has recently been devoted to establishing effective means of destroying pathogenic organisms in the air of premises. Ultra-violet radiation has been applied for the purpose, e.g. in surgical theatres, hospital wards, schools, dormitories, nurseries, cinema theatres, etc. A convenient method is to irradiate circulating air above head-level, so that the occupants are protected from the rays. Many workers have brought forward evidence that ultra-violet radiation of the air of such premises has reduced the incidence of air-borne infection among the occupants (see Wells *et al.*, 1942; Del Mundo and McKhann, Robertson *et al.*, Hollaender, 1943; Hart, 1937, 1938). Chemical disinfection by various antiseptic sprays has also been investigated and applied practically. The object aimed at is to introduce into the air a fine spray of the disinfectant, which in the concentration used will kill pathogenic bacteria without exerting any poisonous or irritant action. Various substances have been found to be suitable, e.g. hypochlorite solutions (yielding hypochlorous acid), certain coal-tar or phenolic derivatives (e.g. resorcinol), glycols, lactic acid, incense and smouldering cardboard. Hypochlorite disinfection has been fully studied with a view to elaborating a practicable and effective means of using it. It has been controversial whether the chemical acts in the form of very small droplets by its contact with the suspended organisms or whether it acts in gaseous form. One of the proprietary solutions, e.g. 'chlorox' so diluted as to constitute a

1 per cent. solution may be employed. According to Challinor (1943), a single spraying with 11 c.c. of a 1 per cent. solution of sodium hypochlorite per 1,000 c.ft. of air, i.e. 0.4 parts per million, can produce approximately a 33 per cent. reduction of the total bacterial content of the air, and repeated spraying brings about a substantial degree of disinfection; repeated or continuous spraying is, of course, necessary. Efficiency depends on the humidity which must be high for maximum disinfecting action. Propylene glycol has

approximately 90 per cent. reduction of naso-pharyngeal organisms in the air of a crowded room. The optimum relative humidity for the activity of propylene glycol is stated to be from 45 to 70 per cent. Triethylene glycol used in the same way has been found to be quantitatively much more potent than the propylene compound. Harris and Stokes have demonstrated in a children's convalescent home the effectiveness of these glycols in reducing the incidence of the common respiratory tract infections. With reference to all these methods of air disinfection, bacteria in dust particles are more resistant than in droplets or droplet-nuclei.

It should be added that in the prevention of dust-borne infection in hospitals, treatment of the floors of wards with spindle oil and of blankets with liquid paraffin can effect a considerable reduction in the dispersal of dust particles and their associated bacteria (see van den Ende and Spooner).

WATER

In the bacteriological examination of water three lines of inquiry may be followed. First, the number of viable bacteria per cubic centimetre may be estimated. Second, the kinds of bacteria present may be investigated. Third, it may be necessary in the case of a particular organism, if present, to ascertain in what number it occurs.

Methods. Collection of samples. Samples are collected in 8-oz. sterile bottles with ground-glass stoppers or in sterile screw capped bottles of similar capacity.

In the case of water taken from a house tap the water should be allowed to run for some time before the sample is taken, as water standing in pipes in a house is under very favourable conditions for bacterial multiplication.

With river waters it is best to immerse the sampling bottle and then remove the stopper with forceps. Care must be taken not to touch the river bed, as the vegetable matter covering it contains many organisms. When water has to be taken from below the surface of a well or lake, a weighted sample bottle must be used. Several special bottles have been devised for such a purpose. Quite good results are obtained by tying two short lengths of string to the neck and stopper respectively of an ordinary bottle; any required length of string can afterwards be joined to these. A piece of lead is attached to the bottom of the bottle by wires passing round the neck. The whole is then wrapped in paper and sterilised. For use the bottle is carefully lowered to the required depth by the string attached to the neck, the stopper is jerked out by means of the other string and the bottle filled.

When some time must elapse before examination the bottles should be packed in ice; otherwise the bacterial content may alter, and an erroneous idea of the number present will be obtained. Immediately after collection a slight diminution in numbers may be observed, but at any rate after six hours an increase over the initial numbers is manifest. Special insulated boxes are obtainable in which water samples can be kept on ice during transport to the laboratory. In the case of water which has been treated with chlorine or chloramine the bottle should contain several small crystals of sodium thiosulphate which are placed in it before it is sterilised.

Counting of bacteria in water. This is done by adding a given quantity of water to 10 c.c. of melted agar, plating and counting the colonies which develop. The amount of water added depends on its source and varies from 0.1 c.c. of a water likely to have a

high bacterial content to 5 c.c. of a purer water. The plates should be duplicated and one incubated at 37° C. for two days, the other at 22° C. for three days. The medium should be standardised to pH 7.2. The plates incubated at 22° C. give an idea of the numbers of purely saprophytic bacteria present; the plates incubated at 37° C., those of more parasitic nature. As the intestinal bacteria grow at 37° C., the determination of the numbers at this temperature is important. The counts in the two media usually differ according to the prevalence of saprophytic or parasitic organisms respectively. In unpolluted water the number of colonies at 22° C. is much higher than at 37° C., a contaminated water may also yield a higher count at 22° C. but the difference between the counts at low temperature and at 37° C. is less.

Detection of the presence of special organisms (a) *The B. coli group* In public health work the most important inquiry with regard to a water is directed to the investigation of the presence or absence of coliform bacilli. Here the method which has usually been adopted is to determine the smallest quantity of a water which gives evidence of containing these organisms, or their number in a given quantity of water (e.g. 100 c.c.), with reference also to the types of coliform bacilli present.

As the primary culture medium MacConkey's bile-salt lactose peptone water¹ (with neutral-red added) is used. In this medium the typical members of the *B. coli* group produce acid and gas. It is thus necessary to put the medium into bottles containing an inverted tube to indicate gas production (*vide* Appendix). Suitable containers are 3 or cylindrical medicine bottles 4½ in. high by 1½ in. in diameter stoppered with cotton wool. The following quantities of the water are added to a series of such bottles. 50 c.c., 20 c.c., 10 c.c., 5 c.c., 1 c.c., and in the case of specially suspicious waters, 0.5 c.c., 0.1 c.c., and even 0.01 c.c. It is advisable to duplicate the series. The quantity and concentration of the medium in each bottle are so arranged that when the sample is added, the resulting fluid shall be of the concentration of MacConkey's medium as ordinarily prepared. Thus, the bottle to which the 50 c.c. sample is to be added contains 10 c.c. of a six-fold concentration of the medium. For the 20 c.c. sample, 20 c.c. of a medium of double strength, for 10 c.c., 10 c.c. of the double strength medium; and for 5 c.c., 5 c.c. of double strength medium. For smaller samples, 5 c.c. of the ordinary MacConkey's medium may be used.

For each sample it is necessary to have sterile 25 c.c., 10 c.c., and 1 c.c. graduated pipettes.

The bottles are incubated for forty-eight hours, and it is well to read the results at the end of the first twenty-four hours also. The formation of acid and gas in a bottle is generally recognised as presumptive evidence of the presence of members of the *B. coli* group, and the test up to this stage is generally designated the 'presumptive *B. coli* test', but it is usual to investigate further the bacteria giving rise to this change and determine whether they are 'typical' or 'atypical' coliform bacilli. With this end in view, each bottle in which acid and gas are present is shaken, two or three loopfuls are placed on a plate of MacConkey's neutral-red bile-salt lactose agar and spread over the surface as in making successive stroke inoculations. The plates are incubated for twenty-four hours. As typical *B. coli* produces acid in lactose any colonies of such an organism are of a rose-red colour. Representative colonies are then subcultured on sloped agar and used for further tests, gelatin liquefaction, indole formation, fermentation of inositol, the Voges-Proskauer and methyl-red reactions, and the citrate-utilisation test. The characters of a typical *B. coli* on which, in our opinion, special stress should be laid are absence of gelatin liquefaction, fermentation (with acid and gas production) of lactose, absence of fermentation of inositol, production of indole from peptone water, and a positive methyl-red reaction. This subject has been more fully discussed in Chapter XIII.

The final result can be stated in terms of the smallest amount of the sample which contains typical *B. coli*, provided both bottles (in the duplicate series) inoculated with this amount yield the organism and all larger volumes tested also show it. It must be remembered that the method is one of random sampling and irregular results may sometimes be obtained, e.g. a certain volume may yield *B. coli* when the organism appears to be absent from a larger quantity.

A more accurate assessment can be made by testing multiple samples, so that the average result is obtained. Thus, if 15 following quantities, 0.1 c.c., 1 c.c., and 10 c.c., or quantities are tested, the probable number of coliform bacteria in 100 c.c. can be computed according to the combinations of positive and negative results as given in tables compiled by McCrady (see 'The Bacteriological Examination of Water Supplies', Report

¹ Two per cent peptone water with 0.5 per cent sodium taurocholate, 0.5 per cent sodium chloride, and 1 per cent lactose (*vide* Appendix).

No. 71, Ministry of Health, 1939) The range of quantities selected would depend on the probable state of the water; thus in testing a chlorinated water a 50 c.c. quantity would be included, but not 0.1 c.c. quantities.

While the presence of 'typical' or 'faecal' coliform bacilli can be determined by testing the biochemical reactions of the organisms present in the cultures as described above, the Eijkman reaction (i.e. ability of typical coliform bacilli to produce gas from lactose at 44° C., p. 313) has been used by various workers as a simple and rapid method of differentiating 'typical' from 'atypical' organisms. For this purpose two sets of the various quantities (as above) are added to MacConkey's fluid medium, one set being incubated for two days at 37° C., the other at 44° C. (in a water-bath). The former set indicates the *B. coli* content of the water irrespective of the type of organism; the latter the 'typical' or 'faecal' *B. coli* content. An alternative procedure is to incubate the bottles at 37° C. and then make subcultures from those bottles showing acid and gas into tubes of MacConkey's medium, incubating the subcultures at 44° C. for two days.

(b) *B. welchii* and streptococci. The detection of *B. welchii* has been used in water examination as a means of proving the presence of excretal pollution. Fifty c.c. of water are added to 100 c.c. of sterile milk in a stoppered bottle, and after the mixture has been heated at 80° C. for ten to fifteen minutes a layer of sterile liquid paraffin is superimposed to produce anaerobic conditions. The mixture is then incubated at 37° C. for twenty-four to forty-eight hours and observed for the 'stormy clot' reaction (p. 476). The test can be made a quantitative one by adding varying quantities of water to milk.

The streptococcus present in polluted water is of the enterococcus type. This organism grows in the liquid MacConkey's medium used for the *B. coli* test, fermenting lactose without gas production. After the 'presumptive' *B. coli* test (vide supra), 1 c.c. is withdrawn from each bottle showing acid or acid and gas production and mixed with 9 c.c. sterile water in a tube which is kept at 60° C. for fifteen minutes. This kills the coliform bacilli, but the enterococcus survives. With a capillary pipette a drop is then used to inoculate a plate of MacConkey's agar. After incubation the enterococcus can be recognised as small, deep-red colonies and if necessary identified by further examination. This method allows of a quantitative estimate of the streptococcal content of the water.

An alternative method consists in subculturing on lactose-tellurite-agar from the bottles showing acid or acid and gas production after the 'presumptive' *B. coli* test. The enterococcus growing on this medium produces bluish-black colonies. The medium is as follows: 1 per cent. peptone, 0.5 per cent. lactose, 0.2 per cent. anhydrous dipotassium hydrogen phosphate, 0.5 per cent. sodium chloride and 2 per cent. agar with a 1 in 15,000 concentration of potassium tellurite added after sterilisation of the medium.

Water derived from any natural source contains bacteria, though, as in the case of some artesian wells and some springs, the numbers may be very small, e.g. 1 to 100 per c.c. In rain, snow, and ice there are often great numbers, those in the first two being derived from the air. Great attention has been paid to the bacterial content of wells and rivers. With regard to the

content of the water entering the well. Again, if the sediment of the well has been stirred up, a high bacterial count is obtained. Ordinary wells of medium depth contain from 100 to 2,000 per c.c. With regard to rivers, very varied results are obtained. Moorland streams are often fairly pure. In an ordinary river the numbers present vary at different seasons of the year, while the prevailing temperature, the presence or absence of decaying vegetation or of washings from land, and dilution with large quantities of pure spring water, are other important factors. Thus it has been found that the rivers Thames and Lea are purest in summer, and this has been attributed to the fact that in this season there is most spring water entering, and very little water as washings off land. In the case of other rivers bacteria have been found to be fewest in winter. A great many circumstances must therefore be taken into account in dealing with mere enumerations of water bacteria, and such enumerations are only useful when they are taken simultaneously over a

stretch of river, with special reference to the sources of the water. Thus it is usually found that immediately below a sewage effluent the bacterial content rises, though in a comparatively short distance the numbers may markedly decrease, and it may be that the river, as far as numbers are concerned, may appear to return to its previous bacterial content. The numbers of bacteria present in rivers flowing through inhabited districts therefore vary greatly.

The bacterial count has been used in estimating the efficacy of the filter-beds of a town water-supply. These usually remove from 95 to 98 per cent of the bacteria present, and a town supply as it issues from the filter-beds should not contain more than 100 bacteria per c.c. Again, it is found that the storage of water brings about a very marked purification. Thus Houston showed in one series of observations that while 93 per cent. of samples of raw river Lea water contained *B. coli* in 1 c.c. or less, in the stored water 62 per cent. of the samples showed no *B. coli* to be present in 100 c.c. According to Coplans, however, the diminution is not necessarily due to the organisms being killed, the real cause may be the agglutination of the bacteria following on changes in the electrical conductivity which take place in stored water.

Much more important than the mere enumeration of the bacteria present in a water is the question whether they include forms pathogenic to man. The most important of these are the *typhoid*, *cholera*, and *typhus* bacteria, and, in certain circumstances, the *paratyphoid* bacteria. The small numbers which may be present in a dangerous water, the direct isolation of these organisms is often impracticable and in any case unnecessary for the condemnation of an unsafe water supply. For public health purposes we therefore seek for the presence of indirect bacteriological evidence which might point to the contamination of a water by human excreta. If this be found we deduce that the water is dangerous, as organisms from any case of intestinal disease occurring in the catchment area may find access to it. The criterion here adopted is the determination of the number of typical coliform bacilli present in the water. Houston pointed out that, in crude sewage, members of the *coli* group are practically never fewer than 100,000 per c.c. In these circumstances the presence of typical *B. coli* in a water is the best indirect evidence of the possibility of disease organisms of intestinal origin being likely to gain access to that water. It must, however, be clearly recognised that those organisms are only, as it were, indicators of excretal contamination.

The difficulty, however, is that (except in the case of water from artesian wells) if a sufficient quantity be taken, evidence of the presence of *B. coli* will always be found. This arises from the fact that this organism is present also in the excreta of many animals, and in the excreta of birds, and in the excreta of fish, and in the excreta of insects, and in the excreta of plants, and in the excreta of the soil, and in the excreta of the atmosphere, and in the excreta of the water, and in the excreta of the human sources, and the consumption of such a water in any condition, may be perfectly safe. It is therefore impossible to set up any absolute standards of the bacteriological purity of a water based only on the estimation of the numbers of *B. coli* present. In any particular case the results must be considered along with those of chemical analysis and inspection of the locality. The difficulty is greatest when dealing with water derived from sewage-contaminated rivers, from agricultural land, and from surface wells. With regard to the first two sources, the water should never be used in an unfiltered condition, and with regard to the last, every case must be considered on its own merits. In addition to filtration, chlorination of such waters has come to be a usual practice. It may be said that under ordinary circum-

stances an inspection of the surroundings and an unfavourable chemical analysis are sufficient to condemn a water, even if a bacteriological examination showed the absence of *B. coli* in large quantities, and further, if in a suspicious locality the bacteriological analysis yielded a bad result, the water ought to be condemned even when from the chemical analysis it could be applied it may be said that when or less of a water from a suspicious

On the other hand, waters of a high degree of purity show absence of *B. coli* from 50 c c or even 100 c c., and it might be said that in a pure water *B. coli* should be absent from 25 c c. A chlorinated water should be free from coliform bacilli in 100 c c

In the Ministry of Health report (cited above) the following classification of piped water supplies (sampled on entering the distribution system) is recommended.

	<i>B. coli</i> count (presumptive test) in 100 c c
Class 1 Highly satisfactory.	Less than 1
„ 2 Satisfactory	1-2
„ 3 Suspicious.	3-10
„ 4. Unsatisfactory	Greater than 10.

It is pointed out that 50 per cent of samples of unchlorinated water supplies should be in Class 1 throughout the year, that 80 per cent should not be below Class 2, and that the remainder should not be below Class 3, while chlorinated water should be in Class 1. If an unchlorinated water occasionally falls into Class 3 this may not be of special significance, but if there is a fall to Class 4, the water should be considered unsafe. When there is a fall to Class 3, however, the type of coliform bacillus should be ascertained, if the organism is atypical less significance would be attached to the result than if the high count were due to typical organisms. It has been shown, nevertheless, that an increased number of atypical coliform bacilli may be antecedent to a dangerous pollution.

in use are capable, if properly worked, of delivering an effluent which does not yield *B. coli* in amounts less than 100 c c, and such a degree of efficiency should in all cases be aimed at

As *B. coli* is fairly widespread in nature, valuable supporting evidence is found in the presence of *B. welchii* and of streptococci, both of which are constant inhabitants of the human intestine. The spores of the former are numerous in sewage, and the presence of the latter can always be recognised in 0.001 grm. of human faeces

It should be emphasised that in water artificially polluted with sewage containing intestinal bacteria, these can be detected by bacteriological methods in mixtures from ten to a hundred times more dilute than those in which the pollution can be detected by purely chemical methods

must be tested for the purpose. Various methods have been used. Of these the following may be recommended:

to enrich organisms of the typhoid-paratyphoid group while suppressing *B. coli*. After incubation for twenty-four hours

The other enrichment media for the typhoid-paratyphoid bacilli may also be used.

To test a larger volume of water, the sample is passed through a Sertz disk, which is then used as the inoculum of a flask of alkaline peptone water.

SEWAGE

It is sometimes necessary to examine the bacterial content of sewage, especially in connection with the efficiency of purification works. The main lines of inquiry are here the same as for water, the only modification necessary being that the numbers are much higher. *e.g.* a series of counts of the *B. coli* group in sewage, these may vary from a million to ten millions or even more per c.c., and here of course the question of the presence of intestinal organisms of the *B. coli* group is of importance. The numbers of these are large, and members of the group may be detected in 0.000001 c.c. or less. Recently it has been shown that by selective or enrichment methods (*vide* Appendix) organisms of the typhoid-paratyphoid group can be isolated from communal sewage (Wilson, Gray). The numbers of *B. coli* present are frequently considerably reduced by purification methods, but it is to be noted that, even when such methods are most successful, this organism may yet be present in considerable numbers. By no purification method has the production of a potable water been attained, and the high content of *B. coli* in effluents suggests that the passage of typhoid bacilli through a purification system is possible.

The part which bacteria play in the purification of sewage constitutes a question of great interest, to which much attention has been directed

Various methods have been adopted for the biological purification of sewage. One of the earliest methods to be used was the *contact bed*—the sewage coming from the mains was run on to a bed of gravel, clinker, or coke, on which it was allowed to stand for some hours. The effluent was then run out through the bottom of the bed, which was allowed to rest for some hours before being recharged. In a modification of this method the sewage after treatment in a closed tank ('septic tank') is allowed to percolate slowly through a bed consisting of large porous objects, such as broken bricks or large pieces of coke, and here the percolation may be constant, no interval of rest being given. In the explanation given of the rationale of this process, sewage is looked on as existing in three stages: (1) First of all, *fresh sewage*—the newly mixed and very varied material as it enters the main sewers. (2) Secondly, *stale sewage*—the ordinary contents of the main sewers. Here there is abundant oxygen, and as the sewage flows along, there occurs by bacterial action formation of carbon dioxide and ammonia, which combine to form ammonium carbonate. This is the sewage as it reaches the purification works. Here a preliminary mechanical screening is adopted. Sedimentation is also frequently employed at this stage, and in this way 50 per cent of the suspended matter can be separated out as 'sludge'. After this the sewage is run into an airtight tank—the septic tank. (3) It remains there for from twenty-four to thirty-six hours, and becomes a foul-smelling fluid—the *septic sewage*. The chemical changes which take place in the septic tank are of a most complex nature. The sewage entering it contains little free oxygen, and therefore the bacteria which flourish in the tank are probably largely anaerobic, and the changes which they originate consist of the formation of comparatively simple compounds of hydrogen with carbon, sulphur, and phosphorus. As a result, there is a

great reduction in the amount of albuminoid nitrogen, and of solid organic matter. The last is important, as the clogging of ordinary filter beds is largely due to the accumulation of such material, and particularly matter consisting of cellulose. One further important effect is that the size of the particles of the deposited matter is decreased, and therefore it is more easily broken up in the next stage of the process. This consists in sprinkling the effluent from the septic tank on to filter beds, e.g. of coke, where a further purification process takes place. By this method there is first an anaerobic treatment, succeeded by an aerobic, in the latter the process of nitrification occurs by means of the special bacteria concerned. The results are of a satisfactory nature, and there is often a marked diminution in the number of coliform organisms present. In the modern adaptation of this method the crude sewage first enters deep narrow 'grit pits' where stony particles settle out. Thence it passes through screens which remove large objects mechanically and break up fecal masses. The solid matter of the resulting screened sewage, which is all in fine suspension, is either liquefied as far as possible by treatment in septic tanks or else allowed to settle out in sedimentation tanks (to aid this process substances such as lime may be added). The 'tank effluent' is now run through sprinklers, on to 'percolating filters' (bacteria beds) similar in structure to the contact beds mentioned above, while the sludge is periodically removed from the tanks. The filter beds when first constructed have little purifying action, as this depends on the formation, on the constituents of the bed, of a biological film consisting of various bacteria, moulds, algae, and protozoa and harbouring worms, insects, etc. A process of renewal and shedding of the film is constantly occurring at a rate which depends on weather factors, etc. Before the effluent is discharged into a river the solid material is allowed to deposit in 'humus tanks', thereby the nuisance due to fouling of the river bed is minimised.

In the earlier stages of any sewage purification, there is little doubt that the organic material present is being split up by ordinary putrefactive bacteria. In the mains and where open systems of purification are at work, aerobic forms play the chief part, while in the closed methods anaerobic organisms are those chiefly concerned. In contact and percolating systems there is evidence that at first the purifying action of bacteria is materially furthered by physical processes. Thus Dunbar showed that when such a substance as coke is used in a sewage filter bed a considerable amount of the protein material is removed in a very few minutes by adsorption, being of a colloidal nature, it is readily deposited under such circumstances in the pores of the coke in the form of films. After a time such a filter bed becomes clogged, but on access of oxygen being allowed it regains its adsorptive properties—probably from the oxidation of the material adsorbed.

During the purification of sewage, various processes are at work—first, the action of

ably of protozoan and other low forms of animal life, fifthly, it is possible that direct chemical oxidation of the earlier products of bacterial action may occur, and in any case the access of an abundant oxygen supply to adsorbed material hastens its destruction.

The *Activated-Sludge Process* has proved to be an efficient and economic method of sewage purification. Raw sewage is run into long narrow channels in which it is mixed with 15 to 30 per cent 'activated sludge' (derived from previous treatment of sewage) and thoroughly aerated either from a compressed air supply or by mechanical agitation, thereafter it is passed into sedimenting tanks where the sludge separates in the

lying the method are not completely understood, bacterial action plays an important part. The sludge derived from previous treatment of sewage supplies a suitable microbial inoculum for the raw sewage. The conditions of treatment are aerobic and presumably the purification is oxidative in nature and biologically similar to that which occurs in the aerobic contact beds of the older processes (*vide supra*). Physical factors are also concerned and the colloidal material undergoes precipitation and sedimentation.

The effluent from a sewage purification system may contain as many bacteria as the sewage entering, but there is often a marked diminution. It is said by some that pathogenic bacteria do not live in sewage. The typhoid bacillus has been found to die out when placed in sewage, but it may sometimes live in this fluid for a much longer period than that embraced by any purification method. Thus the constant presence of *B. coli*

and other intestinal organisms which has been observed in sewage effluents must still be looked on as significant. The effluent should be disinfected by chlorination before being discharged into a river.

SOIL

The investigation of the bacteria which may be found in the soil is undertaken from various points of view. Information may be desired as to the change its composition

hygienic standpoint, soil bacteria are chiefly of importance in view of the fact that they can be washed out of the soil into water supplies. An important aspect of this question is the significance of certain bacteriological appearances in a water in relation to the soil from which it has come or over which it has flowed.

Methods of Examination. For examination of surface soil or soil near the surface, Houston recommended tin troughs 10 in. by 3 in., and pointed at one extremity, to be wrapped in layers of paper and sterilised by dry heat. If several of these be provided, then the soil can be well rubbed up and a sample secured and placed in a sterile test-tube which many varieties have that of a large gasket-like

instrument:
in it a hollow
mechanism

instrument is bored to the required depth, the door is drawn back, and by varying angles the chamber is filled with earth; the door is re-closed and the instrument withdrawn.

In any soil the two important lines of inquiry are, first, as to the total number of organisms (usually reckoned per grm. of the fresh sample); and secondly, as to the kinds of organisms present. The number of organisms present in a soil is often, however, so great that it is convenient to submit only a fraction of a grm. to examination. The method employed is to weigh the tube containing the soil, shake out an amount of about 1 cc. of the soil, and if shaking, and, if measured from

a graduated pipette are used for investigation. For counting the total number of organisms present in the portion of soil used, small quantities, say 0.1 cc. and 1 cc. of the fluid are added to tubes of melted nutrient agar; after being mixed, the medium is plated, incubated at 22° C., and the colonies are counted after three or four days. From these the total number of organisms (viable in the medium used) in a given amount of soil can be calculated.

to investigate the anaerobic organisms of the

(guinea-pigs, mice)

The numbers of bacteria in the soil determined by the above method vary very much

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public health standpoint it is obviously of importance to know the numbers of organisms indicative of pollution by sewage, e.g. typical *B. coli*, *staphylococci* (see under Water).

For the detection of these bacteria the following procedures may be recommended:
(a) *B. coli* group. A third of a grm. of soil is added to 10 c.c. of broth, shaken up, and loopfuls are spread on one or more plates of MacConkey's neutral-red lactose agar. After twenty-four hours' incubation any red colonies are picked off and subjected to the tests for typical *B. coli*, detailed in Chapter XIII. To ascertain the actual *B. coli* content of the soil, varying amounts of the soil emulsion are added to tubes of MacConkey's fluid medium, as in water examination (q.v.).

(b) *Bacillus welchii* To search for this organism 1 grm of the soil is thoroughly distributed in 100 c.c. sterile broth, and of this 1 c.c., 0.1 c.c., and 0.01 c.c., are added each to a sterile milk tube. These are heated to 80° C. for ten minutes, and then cultivated anaerobically at 37° C. for forty-eight hours. If the characteristic 'stormy clot' reaction is developed, then it may be assumed that this organism is present.

(c) *Enterococcus* As in the examination of water for the enterococcus, the cultures made for determining the *B. coli* content (as above) can be utilised. Cultures in MacConkey's fluid medium which show acid or acid and gas are examined as described on p. 719.

The presence of typical *B. coli* in a soil must be looked on as indicative of recent pollution with excremental matter. The presence of *B. welchii* is also evidence of such pollution, but from the fact that this is a sporing organism the pollution may not have been recent. The presence of the enterococcus may be regarded as evidence of recent excremental pollution, as in the case of typical coliform bacilli.

While such means have been advanced for the obtaining of indirect evidence of pollution of soil, investigations have also been conducted with regard to the viability in the soil of pathogenic bacteria, especially of those likely to be present in excreta, e.g. the typhoid and cholera organisms, etc. The solution of this problem is attended with difficulty, as it is not easy to identify these organisms when they are present in such bacterial mixtures as naturally occur in the soil. Further, bacteria often influence each other's viability in an unfavourable way, and it has been clearly shown that various

for instance, the typhoid bacillus soon dies out in a virgin sandy soil even when it is the only organism present. In experiments made by sowing cultures of *V. cholerae* and certain other pathogenic organisms in plots in a field, it was found that after forty days at the longest, they were no longer demonstrable. Further, strictly pathogenic organisms, even if they remain alive, cannot multiply in soil under natural conditions. In the case of a sporing organism such as the *B. anthracis* the capacity for remaining in a quiescent condition of potential pathogenicity is, of course, much greater.

MILK

The bacteriology of milk presents two aspects, the economic and the hygienic, with the latter of which this chapter is mainly concerned. It may be said that the bacteriological condition of ordinary market milk, as sold in the large communities of this country, is far from satisfactory, and the hygienic aspects of milk bacteriology are therefore of special importance in preventive medicine. In the bacteriological sense, ordinary raw milk is perhaps the most impure of foods, and therefore a potential source of infection. Primarily, cow's milk is a sterile fluid, but from the time it flows along the large ducts of the udder, which may contain bacteria of various types, it becomes progressively contaminated. It is an excellent culture medium, especially for intestinal organisms, and any contamination is therefore progressive unless at very low temperatures. The main sources of the bacteria always found in fresh milk are the external surface of the udder, the hands of the milkers, utensils in which the milk is

about five hundred organisms per c.c., but the bacterial content may be greatly increased under unhygienic conditions, and, on the other hand, it has been shown that the numbers of bacteria may be easily controlled by attention to the cleanliness of the cowhouse, by sterilisation of utensils, by grooming of the animals, washing the udder before milking, etc. There is some evidence that for a short time after milk is withdrawn a slight diminu-

tion of the bacterial content may take place, but before it reaches the consumer, especially in city supplies and in warm weather, the bacterial content of apparently fresh milk may rise to several hundred thousands, or over a million per c c. The organisms present are mainly intestinal bacteria, e g. the *B coli* group, lactobacilli, sporing aerobes and anaerobes, and streptococci, and this is specially significant of the nature of the contamination to which milk is subjected in the process of collection.

The Souring of Milk. Under ordinary conditions the first evidence of bacterial activity, and from the economic standpoint the most important, is the occurrence of souring due to the formation of lactic and other organic acids, and the action of these brings about curdling. The subsequent changes vary with the bacteria present, but ultimately these lead to putrefaction of the ordinary type. The importance of the souring of milk has caused much attention to be devoted to the process, and various associated bacteria have been described.

(a) *Streptococcus lactis*. The features of this organism have been dealt



FIG. 200. *B. acidophilus* from twenty-four hours' growth on agar Gram's method $\times 1,000$

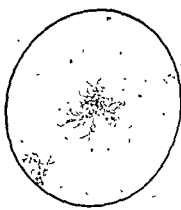


FIG. 201. Surface colony of *B. acidophilus* on agar plate, twenty-four hours' growth $\times 100$

with in Chapter IV. It is an active fermenter of lactose and produces abundant acid from this sugar.

(b) *The lactic acid bacilli (Lactobacilli)* are a group of organisms certain of which occur normally in the alimentary tract of various mammals and are specially numerous in the young animals before weaning. They are non-sporing, non-motile, Gram-positive bacillary organisms; they show great pleomorphism and the same strain may present marked differences in

Some are thermophilic and flourish at 50°C . They do not require oxygen. Among these organisms *B. acidophilus* and *B. bifidus* may be specially mentioned as occurring in the human subject. No pathogenic properties

are attributed to the organism, *B. acidophilus*, a cause of acid residues on the teeth, and the subsequent solution of the dental enamel. The Bacillus Oppler bacillus, another representative of the group, is found in the stomach contents in states in which HCl is absent and lactic fermentation is occurring, e g. gastric cancer. Döderlein's vaginal bacillus (p. 713) also belongs to this group.

Bacillus (or *Lactobacillus*) *acidophilus* is a rather long, stout bacillus ($1\ \mu$ or more broad), with a tendency to curl at the ends, but a more slender form which tends to be in chains also occurs (Fig 200) and short, almost coccoid forms, may be observed. The organism may be recovered from faeces by

under aerobic conditions. The colonies are very minute, and for their detection one must examine the culture under the low power of the microscope, they are of two types, the one a delicate feathery growth somewhat similar (201), the other rounded or fusiform and crab colonies'). Glucose and lactose are reduced but without gas. *B. bulgaricus* originally found in Yoghurt (a fermented milk preparation) is a related organism, it has the same microscopic characters and forms similar feathery colonies, it can be differentiated from *B. acidophilus* by the fact that the

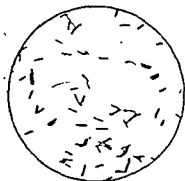


FIG 202, *Bacillus bifidus*, as seen in film preparation from faeces of an infant. Gram's method $\times 1,000$



FIG 203 *Bacillus bifidus*, from a three days culture on agar. Gram's method $\times 1,000$

latter forms acid in 1 per cent maltose broth in forty-eight hours at 37°C whereas *B. bulgaricus* fails to do so, the latter also does not flourish in the human intestine and is thermophilic, growing best between 45° and 62°C .

Bacillus (or *Lactobacillus*) *bifidus* is the predominant organism in the intestine of young breast-fed infants, and in a film of the faeces it appears in practically pure culture (Fig 202), being 4 to $5\ \mu$ long by 0.5 to $0.7\ \mu$ broad, and straight or slightly curved, often with bulbous ends, but in cultures pleomorphism is marked (Fig 203). It was given its specific name as a result of the bifid form it may sometimes assume, which was regarded as characteristic by those who first studied its morphology. Often the bacilli are only weakly Gram-positive. In order to obtain this organism in culture special conditions are required. Deep tubes of 1 per cent lactose or glucose broth neutral to litmus, to which a small piece of sterile rabbit's kidney has been added, are heavily inoculated with a suspension of the faeces and sealed with a layer of sterile vaseline and are kept at 37°C for six to eight days. Any gas which forms at first is expelled by re-melting the vaseline. Stroke cultures are then made on plates of 1 per cent glucose agar or Löffler's serum and are incubated anaerobically. After forty-eight hours *B. bifidus* has formed greyish pin-head colonies which in the next day or two enlarge to 3 to 4 mm in diameter (the only other organisms likely to be present are enterococci which form larger, whitish colonies). Rich subcultures on glucose agar and in

glucose broth will now be found to grow aerobically. Glucose, maltose, saccharose, and certain other sugars are fermented, but without gas production.

A number of similar organisms have been isolated from fermenting milk or carbohydrate materials, some of which produce both acid and gas in the fermentation of sugars.

(c) The third group of milk-souring organisms is the coliform bacilli, certain types of which were originally described in connection with this process (*vide* Chapter XIII). These organisms produce both acid and gas from lactose, and curdling of milk by their growth may be associated with gas formation.

As already stated, there occur in milk a number of bacteria of very different morphological and cultural characters with the common capacity of producing lactic and other acids, and the special qualities of any souring process probably depend on the particular combination of bacteria present. There is considerable evidence that the occurrence of souring holds in abeyance for a time the activity of putrefactive organisms. Many changes, which may be termed economic 'diseases' of milk, are also due to bacteria, e.g. the occurrence of ropy milk, bitter milk, blue milk and 'sweet curdling'.

Pathogenic Organisms in Milk. From the hygienic standpoint the most important consideration is that of the conditions under which pathogenic organisms gain access to milk. These may originate in diseased conditions occurring in the cow, or the milk may become contaminated from cases or carriers of human disease. With regard to the former, the two most important are inflammatory and suppurative disease of the udder, and tuberculosis. Thus chronic mastitis is prevalent and streptococci (*vide* p. 165), or staphylococci are associated with this condition. The milk in such a case may contain pus and blood-stained serum along with large numbers of these organisms. Such milk finds its way into large milk supplies, but it is doubtful if it is responsible for active disease in man, as the common types of mastitis streptococci are infrequently pathogenic to the human subject. Some observers have shown, however, that certain cases of acute bovine mastitis are due to hæmolytic streptococci of human origin. Thus persons harbouring virulent streptococci in their throats may by contact with cows, e.g. in milking, infect the udders; the resulting mastitis may in turn lead to contamination of the milk and spread the infection widely among other persons. It is supposed that scarlatina may also be spread in a similar way by milk. *B. melitensis* may occur in the milk of infected goats, and in certain parts of the world undulant fever is traceable to this source. *B. abortus*, which is closely related to this organism, may be present in the milk of infected cows, and the question of its pathogenicity to the human subject has been discussed in an earlier chapter. In outbreaks of bacterial food-poisoning due to the *Salmonella* group, cow's milk has been found to be infected with organisms of this type and has been responsible for the condition. Cases have been recorded of 'food-poisoning' due to milk and milk products containing staphylococci. The possibility of this originating from staphylococcal infection of the cow's udder must be borne in mind. Human cases of foot-and-mouth disease have occasionally been recorded and traced to milk from infected animals.

Tuberculosis in the cow is a serious source of human tuberculosis arising from the consumption of milk. It should be noted that the disease is exceedingly prevalent in milch cows in this country. The relation of the bovine type of the tubercle bacillus to the human is discussed in Chapter VIII. Here it need only be said that where tuberculous disease occurs in the cow's udder,

tubercle bacilli will be found in the milk, and; further, that where generalised tuberculosis occurs in the animal, tubercle bacilli have been found in the milk without gross evidence of the udder being diseased. The importance of this observation is evident from the fact that a cow with tuberculous lesions in

cattle. Though obvious udder tuberculosis occurs in only about 0.5 per cent of dairy cows, and though only a very small proportion of milk samples from individual cows contain tubercle bacilli, the mixing of milk for market purposes increases the percentage of specimens of tuberculous milk as supplied in the raw state. This percentage may be high, e.g. 6 or 7 per cent or more in the large communities.

Apart from infective conditions of the cow itself, milk may spread infection through its being handled by those suffering from disease or carrying the specific organism. Two diseases occasionally spread in this way are diphtheria and enteric fever. In the case of enteric fever the chief danger lies in the milk being contaminated by a 'carrier'. In the same way milk may be a source of dysenteric infection and cholera in countries in which these diseases are prevalent. There is good reason to believe also that cases and outbreaks of sore throat and scarlatina may owe their origin to milk infected with a hæmolytic streptococcus from a person handling the milk while harbouring this organism, e.g. a convalescent or an unrecognised case. Similarly, a contaminated water used for 'watering' milk or even for rinsing churns, etc., has been known to give rise to milk-borne outbreaks of enteric fever. House-flies infected with organisms of the typhoid-paratyphoid, and dysentery groups may contaminate milk with these organisms, and the same applies to the cholera vibrio in countries in which cholera is epidemic.

A very important bacteriological question is the part played by milk in the causation of infantile diarrhœa. It has been long recognised that cow's milk may be responsible for considerable morbidity and mortality among infants from acute diarrhœa, especially during the summer months when, as might be expected, the bacterial content of milk is generally highest, and without considering any specific bacterium as the causal agent, the question arises whether the ordinary flora of contaminated milk in excessive numbers or the chemical products resulting from their growth, are responsible for such diarrhœal conditions in infants. Specific organisms, however, have been noted in cases and outbreaks of infant diarrhœa, e.g. *B. dysenteriae*, members of the *Salmonella* group, Morgan's bacillus, although milk cannot always be directly incriminated. The etiological problem of infantile diarrhœa is discussed in Chapter XV.

Bacteriological Standards and Graded Milks. Bacteriological standards have now been extensively applied in controlling milk supplies. It may be said that with proper precautions as regards the hygienic collection and distribution of milk, the bacterial content should not exceed 30,000 viable organisms per c.c. The actual factors concerned in

recognised and sold as such, while standards have been laid down for controlling these graded milks. Thus, in the case of the so-called *certified milk* in Scotland the bacterial content must not exceed 30,000 per c.c. and *B. coli* must be absent from 0.1 c.c., this milk must be from tuberculin tested cows and no heat must have been applied to it.

The following standards were laid down under the Milk (1936) Regulations:

England
(accorded
period :
April

Tuberculin-tested (pasteurised) milk must not contain more than 30,000 bacteria per millilitre

Pasteurised milk must not contain more than 100,000 bacteria per millilitre

In 1944 new regulations for 'heat-treated' (including pasteurised) both *Tuberculin-tested (pasteurised)* and *Pasteurised* requirements must conform to certain requirements: *i.e.* must pass a standard phosphatase test (*vide infra*) and must not reduce methylene blue (a test kept in the laboratory at atmospheric pressure between 9 and 10 a.m. in April).

Scotland. *Certified* and *Standard* must not contain more than 30,000 bacteria per millilitre

Heat-treated milk: *i.e.* must pass a standard phosphatase test (*vide infra*) and a methylene-blue test carried out by a standard method and prescribed as follows: the milk must fail to decolourise methylene blue 1 day after the sample has been dehydrated.

shade temperature until the test is completed; in addition, pasteurised milk must contain no coliform bacillus in 0.01 ml. (See: The Heat Treatment of Milk (Prescribed Tests) Order (Scotland), 1944, and The Milk (Special Designations) Amendment Order (Scotland), 1944.)

The Sterilisation and Pasteurisation of Milk. The danger arising from milk being contaminated by disease organisms has caused much attention to be paid to the subject of their destruction before the milk is consumed. The only feasible method is disinfection by heat, and it is fortunate that practically all the important organisms to be considered are non-sporing forms and thus are easily destroyed. The boiling of milk for two or three minutes will kill all harmful organisms, and although some spores may survive, this is by far the most simple and practical method of disinfection for domestic application. Boiling has been objected to, however, on account of the destruction of vitamins, *e.g.* the anti-scorbutic principle; and if very young children on an exclusively milk diet be given boiled milk alone, in a certain number of cases scurvy results. Also, a characteristic taste is imparted by boiling, which is unpleasant to many. Accordingly, to obviate these disadvantages Pasteur's method of heating for twenty minutes at 60° to 80° C. or a modification of this process has been extensively used. This usually kills all but about 5 per cent. of the organism.

tubercle bacillus

Sporing putrefactive

be rapidly cooled, the action of the process as an economic preservative is largely nullified, more especially as the protective milk-souring forms are destroyed.

Pasteurisation has now been generally accepted for the disinfection of milk, and this process has been adopted for commercial purposes and recognised by the Health Authorities under certain conditions, *e.g.* the 'Holder' method in which the milk is maintained at a temperature of 62.8° to 65.5° C. (145° to 150° F) for thirty minutes, and then immediately cooled. An alternative method is the 'High Temperature, Short Time' process in which the milk is heated at 162° F for fifteen seconds and then cooled to 50° F.

¹ The millilitre may be taken as equivalent to the cubic centimetre

The bacteriological standards for pasteurised milk have been given above. It is doubtful whether pasteurisation on a large scale for commercial purposes is uniformly effective, and this is borne out by the bacteriological examination of specimens of pasteurised milk sold as such. The question has also been raised as to whether, in the process of commercial pasteurisation, there is a

of tubercle bacilli (artificially and naturally infected) is rendered non-

recognised method of commercial pasteurisation would thus appear to allow sufficient margin of safety as regards the destruction of the tubercle bacillus provided the pasteurising apparatus is operated with reasonable care.

It may be noted that the *phosphatase test* provides an effective means of controlling the heat-treatment of milk. This test depends on the inactivation at 145° F. within thirty minutes (or 162° F. within fifteen seconds) of the milk enzyme, phosphatase, and a standard method has been elaborated for determining the activity of the enzyme (see Statutory Rules and Orders, No. 349, 1914, H.M. Stationery Office, London). The tubercle bacillus is slightly less resistant to heat than the enzyme, thus, the inactivation of phosphatase in a sample of heat-treated milk would indicate that any tubercle bacilli present had been killed by the treatment.

Methods of Examination.¹ As regards the enumeration of the total viable bacteria in milk and the estimation of the *B. coli* content the methods used in water examination (q.v.) may also be applied. The total content of viable bacteria is estimated as follows: decimal dilutions, e.g. 1/10, 1/100, 1/1,000, 1/10,000—are made with sterile water in sterile bottles. 10 c.c. of these is then plated in standardised nutrient agar and the plates are incubated at 37° C. for two days. When graded milks are examined, particular dilutions may be selected, e.g. 1 in 100 for 'certified' milk, 1 in 1,000 for 'standard' milk. The test should be triplicated so that an average result can be computed. In

tion may be selected, e.g. 1 in 10 for 'certified' milk, 1 in 100 for 'standard' and 'standard' milks (see Regulations cited above). The test for *B. coli* must be triplicated so that an average result can be stated: thus in the case of 'certified' milk, if in two of the three tests acid and gas are absent the specimen may be passed as satisfactory in regard to its content of coliform bacilli.

Methylene blue reduction test. This test, used as a substitute for the enumeration of viable bacteria, depends on the decolorisation of the dye by the bacteria present in the milk, and the degree of bacterial contamination can be gauged by the time in which reduction and decolorisation takes place after methylene blue has been added to a milk sample (*vide supra*). The procedure may be stated briefly as follows: 10 c.c. of the milk sample are placed in a special sterile test tube and 1 c.c. of a standard methylene blue solution is added. The tube is closed with a sterile rubber stopper and the contents are carefully mixed. The tube is then placed at 37° C. in a water bath and observations are made every half-hour. Decolorisation is taken as complete when the milk is decolorised to within 5 mm. of the top of the column. (Modifications of the test applied to heat-treated milk are referred to on p. 730.)

For the detection of tubercle bacilli, a large quantity of the milk, e.g. 100 c.c., is thoroughly centrifuged, and from the sediment guinea-pigs are inoculated subcutaneously. The sediment may be examined microscopically for tubercle bacilli by the usual method but reliance can hardly be placed on the mere finding of acid-fast bacilli, and the

¹ Under the Milk (Special Designations) Orders 1936, for England and Scotland standard methods are described in official memoranda. Reference should be made to these for full details of such methods.

animal test must be employed. The occurrence of tuberculosis in the inoculated animals constitutes conclusive evidence. The lesions should be examined for tubercle bacilli to exclude pseudo-tubercle and other conditions that may simulate tuberculosis, it is noteworthy that *B. abortus* can produce a pathological condition in guinea-pigs

examination of milk in hygiene work, various other procedures have been employed in milk bacteriology; for these, reference should be made to works on agricultural and dairy bacteriology.

APPENDIX—METHODS

THE methods chiefly used in bacteriological work are concerned with demonstrating the presence of bacteria, cultivating them, and studying their actions *in vitro* and *in vivo*. Proficiency can be attained only by practical work in the laboratory, but here an outline of the chief procedures will be given along with details of the composition and preparation of the commonly used culture media and reagents. Bacteria can seldom be identified by their microscopic appearances alone. It is usually necessary to obtain growths, preferably on artificial food media, and when it has been ascertained that such cultures consist of a single species of organisms, i.e. are pure cultures, the following characters should be examined: (a) the naked-eye and microscopic appearances, (b) biological reactions *in vitro*, e.g. fermentation of sugars, production of special chemical products, serological behaviour, and (c) in the case of pathogenic organisms, the production of characteristic disease-effects on inoculation of susceptible hosts.

I

CULTIVATION OF BACTERIA ON ARTIFICIAL NUTRIENT MEDIA

For the study of bacteria either in relation to disease or for other purposes it is important wherever possible, to cultivate the organisms on artificial (dead) nutrient media. Also, when there is a mixture of different species of bacteria these should be separated, so as to yield pure cultures. Accordingly the obtaining of pure cultures is a chief requisite of bacteriological research. Since bacteria are practically ubiquitous, means are needed for destroying all extraneous organisms which may be present in the media, in the receptacles containing the media and on all instruments, etc., which are to come in contact with the cultures. The methods of effecting this destruction are comprised in the term 'sterilisation'. The growth of bacteria in other than their natural surroundings involves also the preparation of sterile artificial food media.

STERILISATION BY HEAT

All bacteria are destroyed by heat. The temperature necessary varies with different bacteria, and the vehicle of heat is also of great importance. The two vehicles employed are (1) hot air and (2) hot water or saturated steam. The former is usually referred to as 'dry heat' the latter as 'moist heat'. As showing the different effects of the two vehicles, spores of *B. anthracis*, which are killed by moist heat at 100° C within one hour, may require three hours dry heat at 140° C to effect death. Originally the various forms of sterilisers were heated by Bunsen burners, now electrical heating controlled by regulators, or steam, is widely used.

DRY HEAT

Red Heat. Bacteriological wires are sterilised by raising them to a dull red heat in the Bunsen flame. The points of forceps and searing irons are treated similarly.

Hot-Air Oven. The oven (Fig. 204) consists of an outer and inner case of sheet iron. In the bottom of the outer there is a large hole. A Bunsen is lit beneath this and thus plays on the bottom of the inner case, round all the sides of which the hot air rises and escapes through holes in the top of the outer case. A thermometer passes down into the interior of the chamber, half-way up which the bulb should be situated. It is found, as a matter of experience, that an exposure in such a chamber for one hour to a temperature of 160 to 170° C is sufficient to kill all the organisms which usually pollute articles in a bacteriological laboratory though circumstances might arise where this would be insufficient. It is important to have the glass vessels dry and to put them into the oven before heating it, and not to open the door after sterilisation till the temperature has

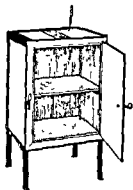


FIG. 204 Hot-air steriliser (simple form)

fallen, -
stopper
stopper

glass to crack. When a glass-
r should be placed between the

This means of sterilisation is used for glass apparatus, *e.g.* flasks, test-tubes, Petri dishes, and pipettes. Such pieces of apparatus are thus obtained sterile and dry.

Cotton-wool plugs are not damaged by the temperature employed and should be inserted before heating. The cotton-wool must be of a suitable quality; non-absorbent wool or inferior grades of absorbent wool, when heated above 140°C often give off volatile products which form a film on the interior of the tubes and inhibit the growth of organisms such as pneumococci. India-rubber washers, etc., are damaged by dry heat. The method is manifestly unsuitable for culture media which contain water.

Oils (almond oil, olive oil, and liquid paraffin, etc.) are sterilised by heating at 150°C for one hour.

Flaming. Small objects may be sterilised by 'flaming', *i.e.* passing them repeatedly through the flame; this method is used for cover-slips, slides, the mouth of culture tubes, cotton-wool stoppers, paper, etc.; but care must be taken not to melt or char the objects.

MOIST HEAT

Boiling Water. Five minutes at 100°C is sufficient to kill all non-spored organisms but some spores may resist boiling for over an hour. This method is useful for tubes, syringes, instruments, etc. The form of steriliser commonly used is an enamel-ware fish-kettle pattern with lid, which should be provided with a removable tray with a raised edge, to prevent articles from falling off. To minimise rusting of knives and steel instruments it is well to boil the water for some time before placing them in it. Distilled or soft water should be used, as hard water produces a deposit on articles boiled in it.

Steam at 100°C . This is the most useful means of preparation and sterilisation for certain culture media. The apparatus ordinarily used is 'Koch's (or Arnold's) steam steriliser' (Fig. 205) or a 'steamer'. In its simplest form this consists of a tall metal cylindrical boiler provided with a lid which has an aperture to permit of the escape of steam, and through which a thermometer projects with its bulb in the interior. A perforated diaphragm is fitted inside at a little distance above the bottom, and water to the depth of several inches is placed in the interior and heat applied by a gas burner underneath. The water quickly boils, and the steam streaming up surrounds flasks, etc., standing on the diaphragm. Hence no evaporation takes place from any medium in the flasks, as these are surrounded by an atmosphere saturated with water vapour. It is convenient to have the cylinder tall enough to hold a litre flask with a funnel seven inches in diameter standing in its neck for filtering medium. The Koch's steriliser may be heated by passing into the water steam from a steam-heating system (if the Koch is attached to the water supply by a ball cock the water in it will be maintained automatically at a constant level).

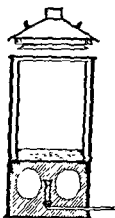


FIG. 205 Koch's
steam steriliser
(simple form) in
section

from the time boiling commences in the water in the steriliser. At any rate, care must always be made for the time required to raise the temperature of the medium to that of the steam surrounding it. It is advisable to cover cotton-wool stoppers of flasks and tubes with several layers of parchment or 'kraft' paper to prevent excessive wetting.

One and a half hours' steaming will usually sterilise any watery fluid. In the case of medium containing gelatin such an exposure is not practicable, as, with long boiling, gelatin tends to lose its physical property of solidification; also prolonged heating may alter labile substances in the medium, *e.g.* certain sugars. The method adopted in this case is to steam for twenty minutes on each of three succeeding days. This is a modification of 'Tyndall's intermittent sterilisation'. The fundamental principle of the method is that all bacteria in a non-spored form are killed by the temperature of boiling water, while if in a spored form they may not be killed. Thus by the sterilisation on the first day all the non-spored forms are destroyed—the spores remaining alive. During the twenty-four hours which intervene before the next heating, these spores, being in a favourable medium, are likely to assume the non-spored form. The next heating kills

these. In case any may still not have changed their spored form, the process is repeated on a third day. Experience shows that usually the medium can now be kept indefinitely in a sterile condition.

Saturated Steam at High Pressure in the Autoclave. Saturated steam at a temperature above 100°C is a rapid means of sterilisation. It is effected in an autoclave (fig. 20b). This is a gun-metal cylinder surrounded by a cylindrical sheet iron case, its top or end, according as it is placed vertically or horizontally, is fastened down with screws and nuts and rendered airtight by a washer. There are a safety valve, tap, and pressure-gauge. The contents are supported on a perforated diaphragm. The source of heat is a large Bunsen burner or electricity or steam from a heating system. In order to prevent cotton-wool stoppers from becoming wet they should be covered with several layers of parchment or 'kraft' paper. The following pressures in lb per sq in (in addition to the 15 lb due to ordinary atmospheric pressure) correspond to the temperatures shown, 3 lb = 107.8°C , 5 lb = 109°C , 10 lb = 116°C , 15 lb = 121.5°C , 20 lb = 126°C . The desired temperature is maintained by adjusting the safety valve so as to blow off at the corresponding pressure. One exposure of media to a temperature of 115° to 120°C for a quarter of an hour is sufficient to kill practically all organisms or spores.

Certain precautions are necessary in using the autoclave. Except where the heat is derived from circulating steam, care must be taken to ensure the presence of sufficient water, so that there is a residuum when steam is fully up, otherwise the steam is superheated and the pressure on the gauge does not indicate the temperature correctly. The procedure in using the autoclave is as follows. Insert the materials to be sterilised; then fix down the lid and apply the heat. Leave the tap of the exhaust valve open until a steady jet of steam escapes from it, since if all the air is not expelled, a mixture of air and steam being present, the pressure shown by the gauge will not accurately indicate the temperature. Then close the tap and reckon the time of sterilisation from the point when the gauge shows the required pressure. When medium is to be sterilised in bulk, however, time must be allowed for attaining the required temperature; the same is the case with masses of fabric, e.g. surgical dressings, which must be completely penetrated by the steam. The requisite information is got by placing recording mercury thermometers in the centre of the material to be sterilised. After ceasing to apply heat, it is necessary to let the apparatus cool well below 100°C before opening it or allowing steam to blow off, otherwise there will be a sudden development of steam when the pressure is removed, and fluid media will be blown out of the flasks.

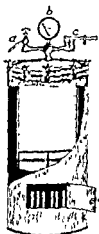


FIG. 20b. Autoclave.

- a Exhaust valve
- b Gauge
- c Safety valve

Autoclaving is the best method of rendering infected material innocuous, such as old cultures, especially when spores are present. It may also be used for sterilising test tubes, etc. fitted with cotton-wool plugs followed by drying in the hot-air oven. It should be noted in the case of dry containers which are closed with impermeable stoppers, that the latter must be loosened before autoclaving, so that steam has free access to the interior. The stoppers should be tightened immediately after opening the autoclave. Gelatin or media containing substances which are readily decomposed, e.g. carbohydrates, must not be exposed to temperatures above 105°C maintained for half an hour.

Where sterile media are required on a large scale and a mains steam supply is available, the apparatus of Messrs Manlove, Alliot & Co Nottingham, is recommended. This is provided with a steam jacket and permits drying of fabrics, etc., after sterilisation. The method of operation given by the makers should be followed.

Temperatures below 100°C . Many organisms in a non-spored form are killed by prolonged exposure to a temperature of 57°C . This fact has been taken advantage of for the sterilisation of blood serum or other materials containing protein, which will coagulate if exposed to a principle by exposing it at intervals to room temperature. A temperature regulator survives such treatment process, provided spores are not present.

80°C for two hours on each of two successive days.

Bacterial cultures which are intended for use as vaccines are sterilised by heating in a

does not melt much below 98° C., but which when melted remains fluid to below 45° C. The reaction of the medium is important, since most bacteria prefer a neutral or slightly alkaline reaction. In general, when preparing media the minimum amount of heating should be used, as prolonged exposure to high temperatures damages their nutritive properties. Also avoidance of filtration through excessive thicknesses of paper, cloth, etc., prevents absorption from the medium of factors which promote the growth of delicate organisms (Cole and Lloyd). The value of nutrient media may be seriously impaired by contamination of the ingredients by traces of copper, with the result that, while large inocula of organisms may grow, small ones fail (O'Meara and MacSweeney). Such medium after heating does not become inhibitory until it has been kept for some time. The advantage of methods of preparing media in which the peptone is added directly to the minced meat seems to depend chiefly on the absorption of traces of copper present as impurity in the peptone by the meat particles. It is likely also that the above method of preparation avoids oxidation of the peptone (H. D. Wright). When organisms fail to grow in media prepared as outlined above it is often found that the addition of serum, blood, or starch etc. promotes growth. It has commonly been assumed that the extra materials supply nutrient substances essential for proliferation of the organisms. But it has been shown in certain cases e.g. for gonococcus and meningococcus (Glass and Kennett) or *B. pertussis* (Pollock), that blood in the medium can be replaced by charcoal. This acts in the case of *B. pertussis* in virtue of absorbing fatty acids harmful to the organisms. Accordingly, it must be borne in mind that such additions may improve the nutrient qualities of media by altering or fixing harmful constituents.

Very many different media have been used either where an organism grows with difficulty, or where some special growth characteristic is to be studied. Only the chief of these are given here, others have been dealt with under the respective organisms.

Broth

The flesh of the ox, calf or horse is usually employed. Horse heart has the advantage of being cheaper and containing less fat than the others, though generally quite suitable, it has the disadvantage for certain purposes of containing a larger proportion of fermentable sugar. The flesh must be freed from fat and finely minced. To 500 grms. of mince add 1 000 c.c. distilled water, 10 grms. of a suitable commercial peptone, and 5 grms. of sodium chloride. Mix well and heat for twenty minutes at 68° C., stirring at intervals. Shake well and steam in the Koch for two hours, filter through paper (Babston's 'Whatman No. 1' and Green's No. 401 are suitable) and adjust the reaction to pH 8.0 (p. 742). Steam again for thirty minutes and filter through paper. Adjust the reaction (about pH 7.5), tube and sterilise in the autoclave at 120° C. for fifteen minutes.

The commercial meat extract 'Lab-Lemco' added to water in the proportion of 10 grms. to 1 litre forms a substitute for the flesh in the preparation described above.

Glucose Broth. To the above broth after the final filtration add up to 1 per cent. of glucose. Tube and sterilise in the Koch. To broth containing glucose or other fermentable compounds 2 per cent. of chalk may be added before sterilisation, by this means acid formed as a result of bacterial growth is neutralised.

Glycerol Broth. Add 1.5 to 6 per cent. of glycerol (sp. gr. 1.25) to digest broth (*vide infra*) or to ordinary broth after filtration. This medium is especially used for growing the tubercle bacillus when the products of the growth of the latter are required. The lower proportion of glycerol is generally advisable. Sterilise in the Koch.

Digest Basis for Media

The use of minced flesh partially digested with trypsin obviates the addition of peptone and provides a very suitable basis for media. Contact of these media with copper utensils must be avoided at all stages.

Media prepared with tryptic digests as a basis yield much more luxuriant growths of most organisms than do those prepared from ordinary meat extract with peptone, but the latter are preferable for keeping stock cultures, as they maintain the viability of the bacteria for a longer time. Sugar-free media, e.g. those prepared from 'Lab-Lemco' or casein digest, are improved by the addition of a small amount of glucose (0.15 per cent.).

Horse Heart Digest Broth (short method of preparation—modified from Douglas). Horse heart (freed from fat and fibrous tissue) is minced finely and is mixed thoroughly with water in the proportion of 500 grms. mince to 1 litre water. The mixture is rendered

strongly alkaline to litmus paper with 4 per cent NaOH solution, and is allowed to stand at room temperature for half an hour, when the reaction is again tested, more alkali being added should the mixture be acid; it is now heated in the Koch's steriliser at 80° C for half an hour. Then it is cooled to 40° C and 5 c.c. 'liquor trypsinæ co.'¹ or 1 per cent. of Pancreatin (B D H) and 10 c.c. chloroform are added; the mixture is well shaken several times and is allowed to stand at room temperature for some hours before being placed in the incubator. After incubating at 37° C for twenty-four hours sufficient 10 per cent. HCl is added to render the reaction acid to litmus paper. The mixture, in a flask which should be about two-thirds full, is placed in the cold Koch and the burner is lighted, so that the temperature is raised slowly and the chloroform evaporates completely, finally after filtration the volume is made up to 1 litre, 0.5 per cent NaCl added, the reaction adjusted to pH 8.0, and it is boiled in the Koch for fifteen minutes, again filtered to remove phosphates, and the reaction adjusted to pH 7.5. The broth may be sterilised at 120° C. for fifteen minutes in plugged flasks and stored for the preparation of agar, etc.

Long Digest Broth is prepared as above, but the mixture with trypsin is kept in a loosely stoppered bottle at 37° C. for twenty-one days, after the first ten days' incubation a similar quantity of liquor trypsinæ co. is added. From the commencement the reaction must be tested daily and 4 per cent NaOH solution added as required, since an acid reaction destroys the trypsin and so prevents progress of the digestion. Media prepared with Long Digest are specially useful for obtaining primary cultures of delicate organisms, e.g. gonococcus, meningococcus, and *B. influenzae*, and also for growing the tubercle bacillus. They are also valuable when abundant growths are required, e.g. for the preparation of vaccines.

Casein and Yeast Digest (CCY) Medium (Gladstone and Fildes). This contains neither meat nor peptone. It is prepared from casein hydrolysed by acid, casein digested by trypsin (as a source of tryptophane), with watery extract of yeast and glutamine (both to supply co-factors), sodium lactate and glycerophosphate (as sources of energy, the last acting also as a buffer). The stock solutions keep indefinitely.

(A) To 200 grms. commercial casein add a mixture of 170 c.c. concentrated HCl plus 110 c.c. distilled water, stir quickly with a glass rod to a uniform suspension, autoclave at 120° C for forty-five minutes. When cool add 40 per cent NaOH till neutral (about 180 c.c.), cool again and filter through asbestos-cotton pulp (p. 770). Dilute the filtrate to 1,000 c.c., place in a Winchester quart bottle, add 1 per cent chloroform, shake briskly at once and at intervals. Store in the dark.

(B) To 200 grms. casein ('light white') and 20 grms. sodium carbonate crystals in a Winchester quart bottle add 1,000 c.c. tap water previously warmed to 37° C., shake well and add a further 1,000 c.c. of the warm water; add 100 c.c. suspension of minced

to 7.4 to 7.5

adjust the pH

To 2,000 c.c.

hour, cool, filter, and adjust pH to 7.4 (6 to 7 c.c. 40 per cent. NaOH)

chloroform, etc., as for (A)

(C) Crumble 250 grms. pressed brewers' yeast into 1,000 c.c. of distilled water which is kept boiling, stir constantly till frothing stops (five minutes), filter and store with chloroform as described for (A)

(D) Sodium glycerophosphate, the commercial solid salt

(E) Glutamine (B D H) 0.03 per cent solution in distilled water, sterilise by filtration, this keeps for a short time in the ice-chest. (The stock of crystals should be kept in a desiccator in the ice-chest)

(F) Sodium lactate, commercial 50 per cent. syrup

To 850 c.c. sterile distilled water add of the above reagents, (A) 35 c.c., (B) 15 c.c., (C) 100 c.c., (D) 10 grms., and (F) 10 c.c., shake to dissolve the glycerophosphate. Autoclave at 115° C for twenty minutes and add 5 c.c. of sterile solution (E)

Hartley's Broth. This medium is very suitable for the production of diphtheria toxin. 150 grms. of minced horse muscle are mixed with 250 c.c. tap water and heated to 80° C in the Koch. 250 c.c. of 0.8 per cent solution of anhydrous sodium carbonate are then added, and the mixture cooled to 45° C, after which 5 c.c. of chloroform and

¹ This is made by Allen & Hanburys, 7 Vere Street, Cavendish Square, London, W.1. Hartley found that owing to its containing glycerol, it is unsuitable for media for certain purposes, e.g. for obtaining diphtheria toxin, when trypsin prepared from pig's pancreas should be used, as recommended by Cole and Onslow (p. 739)

5 c.c. of pancreatic extract (Cole and Onslow)¹ are added (the amount of pancreatic extract required varies with different preparations). The mixture is incubated at 37° C. for six hours, the vessel being shaken at frequent intervals. 40 c.c. of normal hydrochloric acid are then added, and the mixture heated in the Koch for half an hour, then cooled and filtered. The reaction of the filtrate is adjusted to pH 8, and the medium distributed into containers. For the sterilisation of small quantities (100 c.c. medium in half-litre bottles) free steam is passed through the autoclave for one hour, then the pressure is raised slowly to 25 lb. and the steam turned off. For larger quantities (1 litre of medium in double Winchester quart bottles) the same method of sterilisation is adopted, except that the pressure is maintained at 25 lb. for half an hour.

Peptone Water

A simple solution of peptone constitutes a suitable culture medium for many bacteria. The peptone in the proportion of 1 to 2 per cent, along with 0.5 per cent NaCl, is dissolved in distilled water by heating. The pH should be adjusted to 7.5 for general purposes. The fluid is then filtered, placed in tubes and sterilised. Peptone water is much used for testing the formation of indole by bacteria, and by the addition of sugars to it the fermentative powers of many organisms may be tested (p. 754) an indicator being added to show any change in reaction. For these purposes it is essential to test the peptone for the absence of sugar and indole beforehand.

Glucose Phosphate Peptone. Dissolve 5 grms. peptone and 5 grms. dipotassium hydrogen phosphate (anhydrous) in 1 litre of distilled water in the Koch. Filter hot through paper, adjust the reaction to pH 7.5 at room temperature, then add 5 grms. glucose, dissolve and mix thoroughly. Distribute in test tubes and sterilise at 115° C. for ten minutes. This is used for testing the Voges-Proskauer reaction of *coliform* bacilli and for the methyl-red reaction.

Cooked-Meat Medium

A medium containing minced heart muscle was introduced by Robertson especially for the cultivation of anaerobes. The following is Lepper and Martin's modification of this medium. 500 grms. of fresh bullock's heart are minced, placed in 500 c.c. of boiling N/20 caustic soda and allowed to simmer for twenty minutes, by the end of which time the neutralisation of the lactic acid will be insured and the pH of the liquor should be about 7.5. The liquid is drained off on a muslin filter, and while still hot the minced meat is pressed in a cloth and allowed to dry partially by being spread on a cloth or filter paper. In this condition it can be introduced into test tubes without soiling them. Enough should be placed in each to occupy about 5 cm. of the tube, and 1 per cent peptone broth, adjusted to pH 7.5, added until the liquid stands 1 cm. above the meat. The tubes must be kept in a bath of boiling water for half an hour to drive off the entangled air, then they are autoclaved at 120° C. for twenty minutes. The inoculum should be introduced towards the bottom of the tube in contact with the meat. A similar medium may be prepared with brain.

Gelatin Media

These are simply broth solidified by the addition of gelatin. Highly characteristic growths of organisms take place on gelatin media which are liquefied by certain species. Gelatin media, however, have the disadvantage of not being suitable when growth is to take place at temperatures above 24° C., as the gelatin melts.

Ordinary Gelatin. Broth or digest 1,000 c.c., with gelatin 100 to 150 grms. The gelatin is added to the broth, the mixture allowed to stand at room temperature for

¹ The fresh pancreas of a pig is at once, on removal from the newly killed carcase, placed in 50 per cent spirit and brought to the laboratory, there it is freed from fat as far as possible and weighed. It is minced finely and three times its weight of distilled water and its own weight of absolute alcohol are added. Shake the mixture well in a large bottle and allow it to stand for three days at room temperature, shaking the bottle occasionally. Strain through muslin and filter through a large folded filter paper. The filtrate, which comes through very slowly, is measured and treated with 1 c.c. of concentrated hydrochloric acid for every litre. This causes the appearance of a cloudy precipitate which settles in a few days and can be filtered off. The fluid keeps for an indefinite period if stoppered, without any additional antiseptic. If desired at once, the extract may be used before adding hydrochloric acid, the function of which is to retard the slow auto-destruction of the trypsin.

fifteen minutes and then thoroughly melted in the Koch (about half an hour's heating suffices). The fluid medium is then well agitated and the pH adjusted to 7.5. At this time the medium is done by first cooling 5 c.c. blood serum (of pieces of cotton or resulting coagulum medium is filtered through Green's No. 9014 or 5001 paper or in a Büchner filter through cellulose wadding.

As the medium must be filtered, this is effected by the funnel being placed in a funnel with a water-jacket which is heated. It is advisable to lay a glass plate over the filter funnel to prevent evaporation or condensation-water dropping into the medium. The reaction should be adjusted after filtration. A litre flask of the finished product ought to be quite transparent. The flask is then plugged with cotton wool and sterilised, best in the Koch by the intermittent method, as prolonged boiling, or heating at too high a temperature causes gelatin medium to lose its property of solidification. The exact percentage of gelatin required depends on the temperature at which growth is to take place. In hot summer weather 15 parts per 100 are necessary. But if the gelatin is too stiff, it will split when punctured by the platinum wire used in inoculating it. For ordinary use in British laboratories 10 to 12 per cent. gelatin is sufficient.

Glucose Gelatin. The constituents and mode of preparation are the same as above, with the addition of 1 to 2 per cent. of glucose before sterilisation.

Agar Media

These have the advantage over gelatin that at blood temperature (37° C.), at which most pathogenic organisms grow best, they are solid. Agar is a carbohydrate derived from seaweed in the form of fibre shreds, or granules. It is soluble in 100° C. and on solidifying it always becomes slightly opaque. It is less characteristic than those on gelatin. It is not liquefied by any of the pathogenic bacteria.

Unfiltered Agar (modified from Huntton). 50 gms. agar powder, 500 c.c. water, 10 gms. peptone, and 5 gms. sodium chloride are dissolved in 1 litre of water. The mixture is heated at 68° C. for twenty minutes in an open pan with stirring, and then boiled for fifteen minutes to promote solution. Solid medium is prepared by adding 10 gms. agar powder to 100 c.c. of the liquid medium.

allowed to cool overnight. The solid cone of medium is turned out, the deposit cut off and rejected. The medium is melted, distributed into flasks, screw-cap bottles, or tubes and sterilised at 120° C. for fifteen minutes in the autoclave. The final reaction should be pH 7.5 to 7.6.

Digest Broth Agar. Agar medium may be made with digest broth as a base. The procedure is similar to that for gelatin.

Glycerol Agar. To the above agar media before sterilisation add 1.5 to 6 per cent. of glycerol and sterilise in the Koch. This is used especially for growing the tubercle bacillus.

Semi-Solid Agar. A semi-solid medium is prepared by adding 10 gms. agar powder to 100 c.c. of the liquid medium. It is used for increasing the motility of flagellate bacteria and obtaining growths of organisms which do not flourish in a fluid medium or on ordinary solid agar. It is prepared by adding to any of the fluid bases 0.3 to 0.5 per cent. of agar and proceeding as usual. It may be used as a thin layer in plates or in deep tubes.

CCY Agar. To 15 gms. agar powder add gradually 850 c.c. distilled water to make 865 c.c. of medium. It is used for the purpose of increasing the motility of flagellate bacteria and obtaining growths of organisms which do not flourish in a fluid medium or on ordinary solid agar. It is prepared by adding to any of the fluid bases 0.3 to 0.5 per cent. of agar and proceeding as usual. It may be used as a thin layer in plates or in deep tubes.

first a smooth paste and finally a uniform suspension. Place in a heated autoclave and steam for five minutes, then raise the pressure to 15 lb and turn off the heat, open in thirty minutes. Heat to 100° C the mixture of reagents (1), (H), (C), (D), and (E) (see p. 736) and mix with the agar solution also at this temperature. Add 3.6 c.c. of 40 per cent NaOH, place in the steamer and ascertain that the temperature is above 90° C. When the precipitate of phosphates has settled filter through asbestos-cotton pulp (p. 770) as rapidly as possible (under ten minutes), the whole apparatus being heated in the steamer and care being taken not to shake up the precipitate. Add to the filtrate at once 2.05 c.c. concentrated HCl (32 per cent, sp. gr. 1.142), check the pH, which should be 7.6, with cresol red as indicator. Add 5 c.c. glutamine solution (L). Distribute in tubes or bottles and autoclave at 115° C. for twenty minutes. Cultures on this medium should be incubated in an atmosphere containing 5 per cent of CO₂.

For the use of this basis in preparing special media, the original paper should be consulted.

Liver Infusion Agar. This medium is specially recommended for the isolation of *Brucella abortus*. Fresh ox liver is finely minced and 500 grms. are mixed with 500 c.c. distilled water, kept in the cold for twenty-four hours, steamed for twenty minutes in a flask, well stirred and steamed for ninety minutes further, then filtered through iron wire gauze. To 500 c.c. of this extract are added 20 grms. washed agar, 5 grms. peptone (preferably Bacto), 5 grms. sodium chloride and 500 c.c. distilled water. The mixture is steamed for one hour in a flask, cooled to 60° C., and the pH adjusted to 7.2 and again steamed for thirty minutes. The medium is decanted into sterile flasks or tubes and autoclaved for thirty minutes at 120° C. The final pH should be 6.6 to 6.8. Reheating of the liver extract should be avoided, the growth-promoting properties being damaged thereby. When there is likelihood of a mixed infection sufficient 0.1 per cent aqueous crystal violet solution should be added to yield a concentration of 1 in 700,000 of dye in the medium.

Silica Gel Medium

Silica gel may be used instead of agar to solidify medium. The following is a method of preparation (Anderson and MacSwen). Sodium silicate (water glass) is diluted with water to sp. gr. 1.3, 1 volume of this solution is mixed with 9 volumes of broth or other fluid medium and 25 c.c. of 0.04 per cent bromothymol blue indicator added per litre. The solution is sterilised by filtration through Ford SB pads immediately after mixing and filled into sterile containers. The pH is then adjusted by adding aseptically 8N or 4N phosphoric acid—the stronger solution being used for larger volumes of medium, e.g. 100 c.c. To bring the broth silicate mixture to a pH of 7.2 as shown by a pale blue-green colour of the indicator, about one-tenth of its volume of 8N acid is required. Gel forms in about two minutes, thus allowing an interval for sloping or plating. Plates of the medium should be incubated in the inverted position for twenty-four hours before use so as to secure a dry surface.

Media to Counteract the Presence of Sulphonamide Compounds and Penicillin

Specimens of blood, pus, etc., from patients under treatment with sulphonamide compounds or penicillin may contain sufficient of these reagents to interfere with the growth of susceptible organisms in the usual media. They are counteracted respectively by *p*-amino benzoic acid and penicillinase.

p-amino benzoic acid is added to medium in a concentration of 1 in 20,000 before sterilisation. Even in the absence of sulphonamide compounds it may promote bacterial growth.

Penicillinase. Various organisms which are insusceptible to penicillin produce an enzyme which destroys it. According to Duthie certain strains of *B. subtilis* are active producers of penicillinase. The mode of preparation is as follows: of a fully grown broth culture of *B. subtilis* No. 6316 (Nat. Col. Type Cults.) 0.4 c.c. is used to inoculate 20 c.c. of 'Lab-Lemco' broth in a 4 oz. 'medical flat' screw-capped bottle, which is incubated at 26° C. lying on its broad side. On the fourth and sixth days of incubation 1,000 Oxford units of penicillin dissolved in 0.4 c.c. are added and the bottle shaken by hand for a few minutes. On the ninth day the medium is neutralised by addition of acid and centrifuged then filtered through a 3.6 cm. Seitz disk. The addition of this filtrate in a concentration of 1 in 600 to broth containing 25 units of penicillin per c.c. and inoculated with *Staph. aureus* should lead to growth in twelve hours at 37° C. Such a preparation of penicillinase when added in a concentration of 1 in 4,000 to fluid media or 1 in 500 in solid media should neutralise much more penicillin than is likely to be present in specimens of blood, pus, etc. from patients under treatment.

THE REACTION OF MEDIA

In general, each species of organisms grows best in media of a particular reaction, and the optimum may lie within narrow limits. Accordingly, the adjustment of the reaction

... of an amphoteric reaction, i.e. one where red litmus is turned blue and vice versa, is thus avoided. The test paper must be immersed in the liquid—the transfer of drops to the paper by means of a glass rod is not satisfactory. Ordinary litmus is not a delicate indicator, however, and, further, no standardisation of the proper tint to be aimed at is possible. . . . the pH of the medium is adjusted usually 7.5, but most pathogenic bac 7.8

Estimation of Hydrogen-ion Concentration (pH). Electrolytes in aqueous solution tend to dissociate into ions. The true acidity of any fluid depends on the number of free or dissociated hydrogen ions which it contains, or, as it is ordinarily expressed, on the hydrogen-ion concentration in relation to the standard, which is 1 gm. of free hydrogen ions in 1 litre. The greater the number of free hydrogen ions the greater is the acidity; the stronger an acid is, the larger is the proportion of its hydrogen in the free or ionised state. As a matter of fact, however, not all the hydrogen is in the form of free ions. For example, the hydrogen-ion concentration of decinormal hydrochloric acid is slightly less than a tenth of the standard, but when the solution of acid is very dilute nearly all the hydrogen ions are free. In the case of a weak acid, however, the hydrogen-ion concentration is often only a fraction of that of hydrochloric acid. Accordingly, no definite relationship exists between normal, decinormal, etc., solutions of acids and their true acidity.

True alkalinity, in a corresponding fashion, depends on hydroxyl ion concentration, and a fluid is neutral when it contains an equal number of free hydrogen and free hydroxyl ions. Pure distilled water is, of course, such a neutral fluid; only a very small proportion of its hydrogen is in the ionised state, the hydrogen-ion concentration being

10^{-7} , that is, 10^{-7} , and its hydroxyl-ion concentration is, of course, the same. According to the system now ordinarily used, the hydrogen-ion concentration, represented as pH, is expressed by the logarithm of the concentration with its sign changed. Thus, in the case of a neutral fluid, e.g. distilled water, the hydrogen-ion concentration is 10^{-7} ; its logarithm is -7 , and therefore its $\text{pH}=7$. The reaction of a fluid is ordinarily expressed as pH. Owing to the conventional change in the sign, the pH increases as the acidity diminishes, and any pH greater than 7 means an alkaline reaction, that is, the fluid contains more hydroxyl ions than hydrogen ions. For example, blood serum and the tissue fluids, which are slightly alkaline, have a pH of approximately 7.5; and, as a rule, this is the optimum reaction of media for the growth of pathogenic bacteria.

It is necessary to bear in mind that there is an important difference between the acidity of a solution as estimated by the amount of standard solution of alkali necessary to neutralise it and the true acidity, i.e. between the 'titration acidity' and the hydrogen-ion concentration. For example, N/10 acetic acid requires as much alkali to neutralise it as N/10 hydrochloric acid, when tested in the usual way by an indicator; whereas the hydrochloric acid contains several times the number of free hydrogen ions which acetic acid does—that is, is several times as strong an acid.

Buffers are substances which diminish the amount of free ions when they are added to a solution, i.e. the presence of a buffer tends to prevent a change in pH. When, for instance, a certain amount of hydrochloric acid is added to a solution of sodium acetate, sodium chloride is formed with the setting free of acetic acid and water, and the acetic acid is less ionised than the hydrochloric acid, thus the mixture contains fewer free hydrogen ions than the acid added. Amongst substances which act as buffers are various salts, like carbonates, phosphates, citrates, etc., amino-acids and proteins, all these act in the way indicated. Buffer substances are abundantly present in ordinary bacterial media, and thus, even when the true neutral point is known, the desired pH cannot be obtained merely by the proportional addition of a standard solution of acid or alkali. Accordingly, buffers are of great importance in relation to the pH of solutions.

Method. The pH of a solution can be directly determined only by means of electro-metric methods. Instead, a colorimetric method which gives fairly satisfactory results is ordinarily used. It depends on the fact that when indicators are changed in colour by the reaction of a medium, there are two points in pH between which the change takes

place and that a particular tint given by an indicator corresponds with a definite pH. The procedure is thus to determine what amount of alkali is necessary to bring a given amount of the medium to a tint corresponding with the desired pH. Alkali is then added to the whole volume of the medium in proportionate amount. Indicators are available which show a colour change over different ranges of pH; there is also a 'universal indicator' mixture,¹ and indicator papers for a range of pH 2 to 10.²

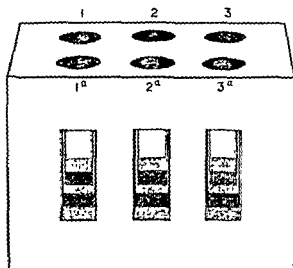


FIG. 207. Comparator used in estimation of hydrogen-ion concentration.

Solutions of graded pH can be prepared by mixing M/15 disodium phosphate (Na_2HPO_4) and M/15 mono-potassium phosphate (KH_2PO_4) in varying proportions.

Phosphate buffer solutions, as well as being used along with indicators for matching the pH of media, are also used for various staining methods. The following mixtures yield the pH's shown with sufficient accuracy for these purposes.

$\text{Na}_2\text{HPO}_4 \cdot 12 \text{ H}_2\text{O}$ M/15 soln (23.96 grms in 1,000 c.c.)		KH_2PO_4 M/15 soln (9 grms in 1,000 c.c.)	pH (approx.)
49.5	plus	50.5	6.8
61.0		39.0	7.0
72.0		28.0	7.2
80.5		19.5	7.4
86.5		13.5	7.6
91.3		8.7	7.8

Such solutions with a suitable indicator added, say phenol red, can be purchased.³ They are supplied in hard white glass tubes—the so-called 'cordite' tubes—of uniform diameter and thickness of glass. And similar tubes are used in making comparative estimations. For example a series of tubes of pH 6.8 to 8.4 (with differences of 0.2) with phenol red show variations in tint from pale yellow to crimson. We have in the first place, to ascertain how much alkali has to be added to a given quantity of medium—5 c.c., in order to bring it to the colour of the desired reaction, say pH 7.5. In such a case it is convenient to use two tubes with pH 7.4 and 7.6 respectively, and to bring the tint of the medium to a point intermediate between the two. A comparator rack to hold two rows of tubes is used and is so arranged that each pair of tubes (front and back row) can be superimposed and examined by light transmitted through them. The scheme of such a comparator is shown in Fig. 207. In 1 and 3, two standard tubes with pH 7.4 and 7.6 are placed, each contains phenol red to show the corresponding tint.

¹ Supplied by British Drug Houses, London. Test paper is also obtainable from Johnson and Sons, Hendon, London.

² Colour standards are not permanent and should be checked at least once a year.

In front of each in 1a and 3a a tube of medium is placed, in 2 a tube of distilled water, and in 2a a tube containing medium and solution of phenol red (*vide infra*). Thus the light transmitted through each pair of tubes passes through the same constituents, namely (a) medium, (b) water or colourless solution, and (c) phenol red; the difference being that in the case of the standard tubes the indicator is in the phosphate solution, whereas in the tube to be tested the indicator is in the medium. The alkali to be added is in the form of N/20 sodium hydroxide solution containing phenol red. This is prepared by taking 500 c.c. of N/10 NaOH, adding 45.5 c.c. of 0.02 per cent. phenol red, and making up to 1,000 c.c. with distilled water. A microburette is necessary to deliver and measure the solution of alkali: for this purpose a 1 c.c. pipette graduated to 0.01 c.c. may be used; a piece of glass tube drawn out to a fine point is attached to the lower end by a piece of rubber tubing with a pinchcock on the rubber to control delivery.

Before standardising, it is convenient to bring the reaction of the medium to about pH 7 by the addition of sodium hydroxide, for example to a reaction which just gives a fine pink with coralline (rosolic acid) paper. Of this medium place 5 c.c. in a cordite tube and add to it 0.5 c.c. of 0.01 per cent. phenol red solution.¹ Note the tint as seen in the comparator, and then gradually run in the N/20 NaOH solution till a tint intermediate between the tints of the two standard tubes is obtained. Repeat the process and take the mean of the two observations. Let the number of c.c. of N/20 NaOH solution = X; then $X \div 20 =$ the number of c.c. of a normal NaOH solution, and $\frac{X}{20} \times \frac{1,000}{a} =$ the number of c.c. of normal NaOH necessary to bring a litre of medium to the required reaction, namely, pH 7.5.

Instead of the series of standard tubes described above, a Lovibond Comparator² may be used, in which measured quantities of the indicator and medium are mixed, and the colour is matched by looking through standard-coloured glasses behind which a similar tube of the medium (without indicator) is placed.

The description given applies to a fluid medium, such as broth. It is best to sterilise the medium before standardisation, and after the reaction has been adjusted, subsequent sterilisation should be carried out at 100° C. Even in this case heating tends to raise somewhat the hydrogen-ion concentration, and a tube of the final product should be tested by adding the indicator. Gelatin may be treated in a similar way to that described, the medium being first liquefied and kept at a temperature of about 30° C.

In the case of agar it is troublesome to carry out the method as directed, owing to the temperature necessary to keep the medium liquid. The procedure usually followed is to bring the broth to the required reaction and then to add the agar and liquefy at 100° C. The agar should be previously brought to a fine state of division and well washed in water, if this is done the agar has usually only a trifling effect on the reaction. Here, again, a test of the reaction of the final product should always be made. This may be done by diluting 0.5 c.c. of the melted medium with 4.5 c.c. of hot neutral distilled water, adding 0.5 c.c. of 0.01 per cent. phenol red solution, allowing the mixture to cool and then comparing the colour with the standards.

RECLAIMED AGAR

Agar which has been employed for growing organisms can be rendered fit for use again by the following method to make sure that the result is such

- 1 One litre of used agar which has been sterilised in the autoclave, as usual, in the Koch at 100° C., adjust the pH if necessary.
- 2 add 10 grms. animal charcoal powder *technical*, B D H and steam at 100° C. for ten minutes, cool to 60° C.
- 3 add 50 c.c. sterile serum (horse, sheep, or ox), steam at 100° C. for one hour; filter through Green's 904½ filter-paper.
- 4 add 50 grms. peptone which has been dissolved in 100 c.c. nutrient broth (and 2.5 grms. of maltose), sterilise in the autoclave.

The addition of maltose is optional, its main purpose being to increase pigmentation of *Staphylococcus aureus*, which is usually poor on reclaimed agar. Blood plates may be made with the reclaimed agar.

¹ Made by diluting a 0.02 per cent. solution in 60 per cent. alcohol with an equal volume of distilled water.

² Supplied by British Drug Houses, London.

DISTRIBUTION OF MEDIA

Media after preparation and before sterilisation, may either be placed in flasks or screw-capped bottles in bulk to be distributed in smaller amounts as required later, or else so distributed at once. The culture of bacteria is usually carried out in test-tubes (conveniently 6 in. by $\frac{1}{2}$ in., but for many purposes smaller tubes, 4 in. by $\frac{1}{2}$ in., are equally suitable and more economical), also in screw-capped bottles ('Universal Containers'), Petri dishes, and, where larger amounts of growth are required, in Kille flasks, etc. Before use all glassware should be thoroughly cleansed (p. 771) and usually sterilised. For protecting the sterile contents of flasks and tubes from contamination with the bacteria of the air cotton-wool plugs are used. If media are to be kept unopened for longer than several weeks the plugs should be flamed and at once covered with sterile kraft paper, since in a damp atmosphere moulds may grow down through uncovered plugs. A medium thus protected will remain sterile for years. Whenever a protecting plug is removed for even a short time the sterility of the contents may be endangered. An advantage of placing media in their final containers directly after preparation is that repeated sterilisations are avoided.

In filling tubes it is convenient to employ as a reservoir for the medium a large glass funnel to which a glass nozzle is attached by rubber tubing. Care must be taken to run the liquid down the centre, so that none of it drops on the inside of the upper part of the tube with which the cotton-wool plug will be in contact; otherwise the latter will subsequently stick to the glass and its removal will be difficult. In the case of liquid medium test tubes are filled about one third full. With solid medium the amount varies according to the purpose for which it is to be used. In the case of gelatin, tubes filled one third full and allowed to solidify while standing upright, are the most commonly used. With organisms needing an abundant supply of oxygen the best growth takes place on the surface of the medium and so for practical purposes the surface ought to be as large as possible. To this end tubes of sloped medium are used (Fig. 208 b). To prepare these tubes are filled only about one sixth full and after sterilisation are allowed to solidify lying on their sides with their necks supported so that the contents extend 3 to 4 in. up, giving an oblique surface after solidification. Thus agar is commonly used in such sloped tubes (gelatin less frequently), and this is the position in which blood serum is inspissated. Agar to be used for making 4 in. Petri plates should be tubed in amounts of 12 to 15 c.c.

Automatic filler—*4-lying's model*¹ enables constant amounts of medium to be delivered rapidly. A large glass funnel acts as the reservoir for the liquid medium (broth, melted agar, etc.). It is connected by indiarubber tubing with a three way stop cock, which is also attached to an all glass 15 c.c. syringe. The syringe is clamped vertically and an adjustable stop placed above it regulates the height to which the plunger may be withdrawn and so fixes the volume which may be taken up by the syringe. The tap of the stop cock is turned to the position in which the interior of the syringe communicates with the funnel. The head of the fluid in the latter then causes the syringe to fill until the plunger meets the stop. Then the tap is turned so that the funnel is cut off from the interior of the syringe but the latter now communicates with the nozzle of the stop cock, whereupon the contents of the syringe are forced out by descent of the plunger. To begin with, air bubbles should be removed from the syringe by filling and emptying it several times by hand.

Tubes of fluid media are conveniently stored on a shelf with a sloping back, to prevent their falling over and the contents spilling into the plugs. Tubes especially of the less commonly used media, should be placed in large jars provided with loose lids, or the medium be kept in bottles provided with screw-caps, otherwise the contents tend to evaporate.

For a method of distributing culture media under sterile conditions, *vide* p. 769

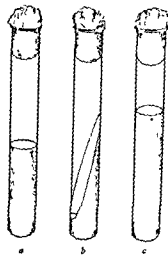


FIG. 208. Tubes of media

- a Ordinary upright tube
- b Sloped tube
- c 'Deep' tube for cultures of anaerobes

¹ Supplied by R. B. Turner & Co., 9-11 Eagle Street, Holborn London W.C.1

SPECIAL MEDIA

Serum and Blood Media

METHOD OF COLLECTING BLOOD FOR OBTAINING SERUM. Blood from a horse, ox, or sheep, obtained at a slaughterhouse, is suitable for most purposes. After the neck vessels have been cut and the blood has been flowing for some time (the first blood is

overnight, preferably in the ice-chest. The serum is then pipetted off. If there is any admixture with red blood corpuscles, the latter can be removed by centrifuging. With care sterile serum can be obtained. When it is desired to use the fluid serum as a culture medium it should be heated as described on p. 735, and finally should be tested for sterility after incubating for forty-eight hours at 37° C. Serum may also be sterilised by filtration (p. 768). Serum sterilised by chloroform may be used in preparing certain media, e.g. Löffler's.

Blood which is to be defibrinated is collected in the same way, but glass beads are placed in the receiving bottle before it is sterilised and the bottle is partially filled. It is then stoppered and shaken continuously for five to ten minutes.

Intubation. In the case of a sheep, the wool is clipped, the skin shaved, cleansed, and sterilised. The vein is made prominent by pressure on the side of the neck lower down. To reduce the chance of contamination a waterproof bag should be placed over the animal's head to begin with. For a horse the procedure is the same except that a small incision in the skin should be made over the vein with a sharp knife.

Being strapped in. The chest-wall is shaved, cleansed, and the needle is passed into the heart; blood is then sucked into the pipette (50 c.c. per kilo of body-weight can be obtained), and is defibrinated in a sterile flask with glass beads. Animals which have been bled must be provided with ample water to drink afterwards.

Small quantities of sterile blood may be obtained from the marginal vein of the rabbit's ear by the following procedure. The area is shaved and cleansed with alcohol and ether; when the skin is dry, melted vaseline (previously sterilised by heating) at a temperature of about 54° C. is smeared thinly with cotton wool on both sides of the ear round the vein. At the base of the ear the vein has been rendered turgid, it is incised. Care is taken that the blood

For other details vide p. 507.

Blood from the human subject is obtained by venepuncture. Ascitic and hydrocele

The more slowly inspissation is performed. The apparatus used for the purpose is one of the various forms of serum inspissator, the temperature being controlled by a regulator.

Coagulated Serum. For most purposes this can be substituted for inspissated serum. A sufficient quantity of serum is placed in a series of sterile test-tubes, these are then placed in a sloped tray, and heated in the Koch at 75° C. for an hour. If the process of heating is carried out too quickly, bubbles of gas are apt to form and to tear up the surface of the medium. This can be avoided if the serum is solidified high up in the Koch at 75° C. for two hours on each of inspissation and sterilisation, at the serum need not be

obtained with special aseptic precautions.

1 Sterile oxalated horse blood, also horse serum may be obtained commercially from Burroughs Wellcome.

Lömer's Blood Serum. This medium is specially suitable for the growth of *B. diphtheriae*, and may be used for other organisms. It has the following composition. Three parts of calf's or lamb's blood serum are mixed with one part 1 per cent glucose veal broth. It can be made from ox or sheep serum and ordinary glucose broth without its qualities being markedly impaired. The method of preparation is otherwise the same as that described for Coagulated Serum. If the medium has to be stored universal containers should be used.

Media Enriched with Blood or Serum

Blood Agar. Defibrinated or oxalated blood is warmed to 45° C., and added to agar of the same temperature in the proportion of 5 to 10 per cent, thoroughly mixed and poured into plates or sloped. Such medium should be incubated before use to ensure that it is sterile.

Blood agar plates may be prepared with a saving of blood as follows. Pour into the plate a thin layer of plain melted agar (about 7.5 c.c. for a 4 in. plate), when this has set, pour on top of it a similar amount of 10 per cent blood agar.

Blood-Smeared Agar. This medium was introduced by Pfeiffer for growing the influenza bacillus, and it may be used for organisms which do not readily grow on the ordinary media. Sloped tubes of agar are employed. Cleanse a finger first with 5 per cent lysol dry, and then wash with absolute alcohol. Allow the alcohol to evaporate. Prick with a needle sterilised by heat and, catching a drop of blood in the loop of a sterile platinum wire, smear it on the surface of the agar. Then keep the tube sloped for about ten minutes until the blood has coagulated. Incubate the tubes at 37° C. before use, to make certain that they are sterile. Animal blood may also be used. Agar poured out in a thin layer in a Petri dish may be smeared with blood in the same way and used for cultures.

Blood Broth. The addition to broth of 10 per cent uncoagulated sterile blood, e.g. rabbit's, yields a fluid medium in which many delicate organisms will grow.

Boiled Blood Agar (Chocolate Agar) is suitable for obtaining cultures of *B. influenzae*, *meningococcus pneumoniae*, etc. To 5 c.c. melted agar medium at 60° C. add 0.5 c.c. (about 10 drops) blood—rabbit's blood is suitable. Heat the mixture by immersing the tube in boiling water for one minute (not longer), then allow to solidify in the sloped position. A clear medium with similar properties is got by centrifuging or filtering out the coagulum before solidification. **Boiled blood broth** is prepared similarly.

Peptic Blood Digest Media (Idles). Keep a mixture of 150 c.c. saline, 6 c.c. concentrated HCl, 50 c.c. defibrinated sheep's blood, and 1 grm. pepsin (B.P. granulated) in a well stoppered bottle at 55° C. in the water bath for two to twenty-four hours. Then add 20 per cent NaOH solution till a sample of the mixture diluted with water gives a permanganate red colour with cresol red indicator—about 12 c.c. are required. Now add concentrated HCl drop by drop till a sample gives almost no change of colour with cresol red, but gives a red tint with phenol red—avoid excess of acid. 0.25 per cent of chloroform is added to the mixture and dissolved by shaking. This digest keeps for many months, before use the mixture should be shaken and a portion equivalent to 2 to 5 per cent added (after heating at 55° C. for thirty minutes to remove the chloroform) with a sterile pipette to broth or agar. These media are useful for the growth of Pfeiffer's bacillus anaerobes etc.

Bordet and Gengou's Medium. An extract of potato is first prepared by adding two parts of water containing 4 per cent of glycerol to one part of diced potato, the mixture is then boiled and the fluid is strained through gauze. One part is mixed with three parts of a 4 per cent solution of agar in 0.6 per cent sodium chloride solution, and the pH is adjusted to 7.5, then the whole is sterilised in the Koch. For preparing 4 in. plates the medium is tubed in amounts of 10 c.c. To a tube of the melted medium at 50° C. 4.5 to 7.5 c.c. of sterile defibrinated rabbit's or human blood (obtained by the method described on p. 807) warmed to 40° C. are added and uniformly mixed. The mixture is then poured into Petri plates which are kept in the ice-chest and may be used until two weeks old. This medium is very suitable for the growth of the bacillus of whooping-cough also for the gonococcus, meningococcus, and influenza bacillus.

Blood-Potato Agar (Kirkpatrick). Many delicate organisms, e.g. *B. influenzae*, *B. pertussis*, the tubercle bacillus, gonococcus, meningococcus, pneumococcus, etc., grow well on this medium. It consists of 100 c.c. Bordet-Gengou potato extract, 300 c.c. Long Digest broth, 10 grms. agar fibre. Dissolve the mixture in the autoclave at 115° C. (thirty minutes), then adjust the pH to 7.5 with 4 per cent caustic soda solution. Fill into 6 in. by 1 in. test-tubes (6 c.c. per tube) and sterilise in the autoclave at 120° C. for fifteen minutes, then cool to 56° C. and add to each tube 0.5 to 1 c.c. fresh sterile defibrinated rabbit blood, mix thoroughly, heat in the boiling water-bath for one minute and slope.

Serum Agar. Certain delicate organisms, *e.g.* gonococcus, require for their growth uncoagulated serum, which should preferably be freshly drawn. Suitable medium consists of agar, especially that prepared with a digest basis; this is melted and cooled to 50° C., then 5 to 10 per cent. of serum is added and, after mixing, the medium is sloped or poured into Petri plates. Usually animal's serum *e.g.* rabbit's serum is used.

Serum-sterile blo

Egg Media

Media containing either the yolk, or both the yolk and the white of egg, have been used for the culture of the tubercle bacillus by Dorset and others. For this purpose the eggs must be fresh, *i.e.* less than a week old. Also cultures on egg medium are made.

the medium into sterile test-tubes or 'Universal containers,' avoiding the formation of air bells, and coagulate by heating in the Koch at 75° C. (the tubes should be placed high up in the steriliser, in a sloping position). Sterilise by heating in the Koch at 80° C. for two hours on three successive days. The medium keeps for several months provided drying does not occur. If the surface of the medium has become dry before inoculation, two drops of sterile broth are placed on the surface. The inoculation material is well rubbed over the surface of the medium (in the case of tubes these are sealed, *e.g.* with a wax disk on the top of the plug). Crystal violet has the property of inhibiting various other organisms which may be present as contaminants. It may be advisable to omit the dye when the medium is intended for cultures of the bovine type of the tubercle bacillus or for other organisms.

Glycerol Egg Medium is prepared as above by using heart digest broth to which has been added 6 per cent. of glycerol.

Löwenstein-Jensen Medium for the Cultivation of the Human Type of Tubercle Bacillus

Salt-starch solution. A solution of the following pure substances¹ is prepared by heating in distilled water

potassium dihydrogen phosphate	0.4	per cent
magnesium sulphate	0.04	"
magnesium citrate	0.1	"
asparagine	0.6	"
glycerol	2.0	"

The solution is steamed in the Koch for two hours and allowed to cool overnight. Then 3 grms. of potato starch¹ are added to 60 c.c. of the solution and the mixture heated gradually in a water-bath with constant stirring for fifteen to twenty minutes until a satisfactory paste has formed, this is kept for one hour in the water-bath at 50° C. after which time it is ready for adding to the egg fluid.

Egg fluid. Fresh hens' eggs (which must be less than one week old) are thoroughly washed in a 5 per cent. solution of soft soap, and then left in running water for one to two hours. The eggs are now broken singly into a sterile vessel (2 to 3 eggs yield 100 c.c.) and the yolks and whites rendered homogeneous by mixing for ten minutes by hand or in a shaking machine, after which the mixture is filtered through sterile gauze, and to 100 c.c. are added 60 c.c. of salt-starch solution prepared as above.

To 160 c.c. of the mixture of salt-starch and egg fluid add 2 c.c. of a 2 per cent. aqueous solution of malachite green in distilled water, which has been incubated for one to two hours beforehand.

The medium is now tubed in 5 c.c. amounts in 1 oz. screw-capped bottles, the caps being tightly screwed on. The bottles are laid on a slope in the inspissator and are heated at 75° C. for half an hour. They are allowed to remain at 37° C. overnight and are heated again on the following day at 75° C. for half an hour. In these bottles the

¹ The ingredients may be obtained from British Drug Houses and specified 'A.R.'

medium will keep for many months, but slopes in test-tubes must be stored in the cold and used within a month

Milk

This is used for observing the effects of bacterial growth in producing coagulation and in fermenting the lactose. It is prepared as follows. Boil well-skimmed milk in the Koch for ten minutes, allow to cool and filter through cotton wool. Add 6 per cent. litmus solution (p. 755) and adjust the reaction. Fill into test-tubes and sterilise for twenty minutes on three successive days. This is *litmus milk*. The litmus may be omitted. The reaction of fresh milk is alkaline. If greater accuracy is required, the initial and final pH and titratable reaction may be measured.

Whey broth and *whey agar* are used for growing *B. acidophilus*. To skimmed milk at 80° to 90° C. just sufficient 10 per cent. HCl is added to precipitate the casein—this is estimated preliminarily several times on a small sample. The fluid is filtered through absorbent cotton wool and then paper, and the pH adjusted to 6.8 to 7.0 with NaOH. Finally 0.5 per cent. peptone is added and the mixture autoclaved at 120° C. for fifteen minutes and filtered to remove coagulum. To this whey broth add 1 to 2 per cent. agar for whey agar.

Potato and Carrot Media

Large potatoes are selected, the surface is cleansed by scrubbing in running water and cylinders are cut with an apple corer or large cork borer, then divided lengthwise by a diagonal cut and washed in running water for at least twelve hours. Each block is placed in a test-tube, the broad end resting on wet cotton wool. Fill the tubes with distilled water until the blocks are completely covered, plug and sterilise by autoclaving for five minutes at 110 to 115° C. on each of two successive days. Pour off the water just before inoculating. Varieties of potatoes which are highly suitable are 'Majestic', 'Epicure', 'British Queen', and 'Arran Comrade'.

Carrot slices treated similarly to those of potato serve as a good medium for the growth of moulds.

Glycerol potato medium. The method of preparation is the same as in ordinary potato medium, except that 6 per cent. glycerol is used in place of the sterile water for filling the tubes.

Potato extract prepared by adding 5 per cent. of potato to water and sterilising the mixture by heat, is valuable for inducing formation of mycelium in certain fungi.

SELECTIVE AND ENRICHMENT MEDIA

Numerous media have been devised for the purpose of isolating a particular organism, e.g. a specific pathogen, from a mixture. They depend upon several principles. In one form (e.g. MacConkey's medium) a substance is added for instance a sugar, which is fermented with the production of acid by certain organisms but not by others of related species, an indicator dye is also incorporated in the medium so that the colonies of the fermenting organisms have a different colour from the non-fermenters. In the case of the coli typhoid group such media usually contain lactose which is fermented by the common coliform bacilli in faeces but not by the specific pathogens. Another class of selective media contains some substance which in the concentration used either (a) specially favours the multiplication of the organism which it is desired to isolate relative to the others, or (b) inhibits the contaminating organisms while permitting the specific pathogen to grow, i.e. a selective antiseptic (e.g. tellurite media for the diphtheria bacillus). For isolating organisms from a mixture with *B. proteus* a reagent is required which inhibits the spreading of the latter, while permitting growth of the others.

Media for the Isolation of Members of the Coli-Typhoid-Salmonella Group

MacConkey's bile salt lactose agar :

sodium taurocholate (commercial)	5.0 grms
peptone (sugar-free)	10.0 "
distilled water (the addition of 0.03 per cent. CaCl ₂ has been recommended)	1 000 c.c.

Dissolve in the Koch at 100° C. for thirty minutes, when cold filter. Add 25 grms. agar fibre (agar powder usually 20 grms.), dissolve in the Koch for thirty minutes, adjust the pH to 7.5, cool to 60° C. and add 50 c.c. serum (sheep, ox, or horse). Steam again in

the Koch for sixty minutes, filter. To each 100 c.c. of filtrate add 1.0 grm. lactose and approximately 0.6 to 1.0 c.c. of 1 per cent aqueous solution of neutral red. Sterilise by the intermittent method.

Note. The bile salt supplied commercially varies in its inhibitory power on bacterial growth, and it may be advisable to employ 0.25 per cent in place of 0.5 per cent originally recommended. The following simple method shows whether the medium is unduly inhibitory: make a slightly opalescent suspension of smooth *B. dysenteriae* (Flexner) and one of *B. typhosus* in broth and from these prepare dilutions of 1:10, 1:100, 1:1,000, etc., then make a stroke of each dilution on a plate of ordinary agar and of bile-salt medium respectively, incubate for twenty-four hours at 37° C., and note the abundance of growth. It will be found that the inoculation with a particular dilution of the suspension gives on agar closely adjacent, but not confluent, colonies; the same dilution should yield a similar abundance of growth on a satisfactory bile-salt medium.

The colonies of any organism giving rise to acid will be of a rose-red colour.

Reclaimed MacConkey's agar

- 1 One litre of used MacConkey's agar, which has been sterilised in the autoclave, is melted in the Koch at 100° C.;
- 2 adjust the pH to 7.5 if necessary (test with indicator paper);
- 3 add 15 grms. animal charcoal powder, technical, B.D.H., and steam at 100° C. for ten minutes, cool to 60° C.;
- 4 add 50 c.c. sterile serum (horse, sheep, or ox);
- 5 steam at 100° C. for one hour, filter through Green's 904½ filter-paper;
- 6 add 0.5 grm. lactose and approximately 1 c.c. of 1 per cent. neutral red solution per 100 c.c. filtrate;
- 7 sterilise in the Koch at 100° C. for twenty minutes on each of three successive days.

Endo's Medium:

ordinary meat extract (or 0.5 per cent of Lab-Lemco in water)	1,000 c.c.
peptone (sugar-free)	10 grms.
sodium chloride	5 "
agar fibre cut into small pieces	20-25 "

Mix the ingredients and heat in the Koch for half an hour; add 10 c.c. of 10 per cent sodium carbonate solution. Cool to 50° C. and add 50 c.c. serum (ox, sheep, or horse), steam in the Koch for one and a half hours, filter while hot through paper. To each 100 c.c. of filtrate add 1 grm. lac of basic fuchsin, and 1.25 c.c. of freshly Sterilise in the Koch for twenty minutes. is hot it appears pink in colour, when medium be pink when cold the amount of sodium sulphite present is insufficient; medium must be kept in the dark. Colonies of lactose-fermenters are red.

These media will yield good results in the hands of those accustomed to them. MacConkey's medium is that most used by British workers, and it has the merit of being easily prepared.

Brilliant Green Enrichment Medium (Browning, Gilmour, and Mackie). Brilliant green has a greater inhibitory effect on coliform organisms in general than on *B. typhosus* and especially the paratyphoid group. The concentration of the dye necessary to bring about such cases as it depends on the number of

0.5 per cent)

free) is used as a 1

a thick
for twelve to
and strokes
Two 4-in
or twenty-
typhoid or

paratyphoid bacilli is obtained from one of the lactis aerogenes, are as resistant to brilliant green as *B. typhosus*, also, in cultures from the faeces of young children 'paracolon' bacilli tend to be enriched by brilliant green.

Tetrathionate Medium (Muller)

- 100 c.c. sterile broth (pH 7.4).
- 5 grms. chalk sterilised in the autoclave.
- 5 grms. sodium tetrathionate crystals dissolved in water to make 10 c.c. and sterilised in the Koch.
- 0.5 gm. iodine ground in a mortar with 0.4 gm. potassium iodide and dissolved in sufficient water to make 2 c.c. —sterilisation is unnecessary.

The constituents are mixed and, without further heating, distributed in amounts of 10 c.c. in sterile plugged test tubes. A tube is inoculated with faeces and, after twenty-four hours' incubation, subcultures are made on a plate of MacConkey's or Ludo's medium. *B. proteus*, when present, tends to predominate in this medium (see Knox *et al.*)

Bismuth Sulphite Agar Medium (Wilson and Blair)¹ contains glucose, sodium sulphite, bismuth ammonium citrate and brilliant green with or without ferrous sulphate. As the preparation of the medium is elaborate, reference should be made to the original description. It has given excellent results in the isolation of *B. typhosus*, *B. paratyphosus* B and *B. enteritidis* (Giertner) from faeces and sewage. The rationale of its action seems to be that brilliant green largely determines its selective effect, while in the presence of glucose *B. typhosus* etc. reduce sulphite to sulphide, which acts on the bismuth, the colonies thus being blackened (Tabelt).

Plates should be poured and used within three or four days.

Bismuth-sulphite powder (K), which is stable in the dry state for several months when kept in a well stoppered bottle, may be added to agar medium just before use (Hobbs, King and Allison). The reagents which are sufficient for 1 litre of finished medium, are placed in a large dry mortar. In order to produce a homogeneous mixture very thorough grinding is required after the addition of each ingredient in the order shown.

bismuth ammonium citrate scales	4.0 grms
sodium phosphate (Na_2HPO_4)	2.5
brilliant green	0.02 gm
ferrous sulphate—dried ($\text{FeSO}_4 \cdot \text{H}_2\text{O}$)	0.45
sodium phosphate (Na_2HPO_4)	2.5 grms
sodium sulphite (Na_2SO_3)	4.0
dextrose	5.0

For one plate to 15 c.c. of melted agar medium add 0.28 gm. of the above mixture which has been heated nearly to boiling point in 1 to 1.5 c.c. water in a test tube. To ensure even distribution, pour several times from one tube to another and then into a 4-in. Petri plate.

Selenite Medium (Leifson) This fluid medium is used for the enrichment of typhoid and paratyphoid bacilli.

sodium hydrogen selenite (anhydrous NaHSO_3)	0.4 gm
monosodium phosphate and disodium phosphate (mixed in the proportion of about 1 to 3—so that the finished medium has a pH of 7)	1.0
peptone	0.5
lactose (or mannitol—Hobbs and Allison)	0.4
distilled water	100 c.c.

(Hobbs and Allison omit the monosodium phosphate and adjust the pH by means of $\text{N}/1 \text{ HCl}$.)

Heat in the Koch for thirty minutes (do not autoclave)—this solution prepared with mannitol keeps indefinitely at room temperature, tube so as to give a column at least 2 in. high. Add 10 to 20 per cent. of a faecal specimen and emulsify, after twenty-four to thirty-six hours at 37°C., subculture on desoxycholate citrate agar. To urine, add an equal volume of double strength medium, or add the dry constituents of the medium to the specimen. For sewage, add nine volumes to one volume of medium prepared as above, but containing 1.5 per cent. selenite. For milk, add nine volumes to one volume of 2.0 per cent. selenite in 10 per cent. peptone water, in the case of a highly contaminated milk dilute with three volumes of sterile water and use nine volumes of this dilution instead of whole milk.

Desoxycholate-citrate Medium (vide *infra*) is also valuable for the isolation of typhoid-paratyphoid salmonella bacilli. It is used as for the isolation of dysentery bacilli.

¹ The medium is obtainable commercially.

Media for the Isolation of Dysentery Bacilli

Media such as MacConkey's and Endo's show the presence of dysentery bacilli when in large numbers. These, however, are readily suppressed by coliform organisms, and the only selective enrichment medium is that of Leifson.

Desoxycholate-citrate Medium (Leifson—modified). 5 grms. Lab-Lemco and 5 grms. peptone (Armour's or B.D.H.) are dissolved in 1 litre of water by heating in the Koch for twenty to thirty minutes; adjust the pH to 8.0 and filter. To 500 c.c. of the filtrate add 20 to 25 grms. agar fibre and dissolve in the Koch.

To the remainder of the filtrate while hot add the following reagents (B.D.H., A.R. grade) in the order shown: sodium desoxycholate 30 grms., sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) 8.5 grms., sodium thiosulphate 8.5 grms., a solution of 1.0 gm. ferric citrate (made by adding the red scale preparation to 40 c.c. water at 60° C. with constant stirring—this takes about thirty minutes), lactose 10.0 grms. To this mixture add the solution of agar, then 2.5 c.c. of a 1 per cent. aqueous solution of neutral red. Distribute in sterile containers or tubes. Melt and plate just before use—it is desirable that the medium should undergo no further heating.

Media for the Isolation of *V. Cholerae*

Blood-Alkali Agar (Dieudonné). This medium, introduced for the culture of the cholera vibrio, for which purpose it has been found extremely suitable, inhibits the growth of most of the intestinal bacteria, e.g., *B. coli* does not grow on it, or does so very slightly. Equal parts of defibrinated ox blood and normal caustic soda solution are mixed and heated in the Koch for one and a half hours and this is repeated on eight successive days, as large a surface as possible being exposed, so as to aid removal of ammonia. After standing further for ten days at room temperature, the solution is ready for use; it keeps for two months. Of this solution three parts are added to seven parts of 3 per cent. agar medium and the mixture is poured into plates, which may be used at once.

Aronson's Medium. Stock solutions in distilled water—(a) 10 per cent. anhydrous sodium carbonate, (b) 10 per cent. sodium sulphite, (c) 20 per cent. saccharose, (d) 20 per cent. dextrin; each is steamed in the Koch for thirty minutes. To 300 c.c. melted 3 per cent. agar medium are added 20 c.c. of solution (a) and the mixture is steamed for thirty minutes, then the following are added—15 c.c. each of solutions (c) and (d), 1.2 c.c. of basic fuchsin solution (3.5 grms. in 90 c.c. absolute alcohol plus 10 c.c. distilled water), and 6.5 c.c. of solution (b); the mixture is steamed for twenty minutes. A heavy precipitate quickly settles, and plates are poured from the supernatant; these may be stored in the ice-chest. Colonies of *V. cholerae* after twenty-four hours at 37° C. have a reddish centre and a pale marginal zone; coliform organisms usually form small faintly pink colonies.

Desoxycholate-citrate Medium and **Bismuth Sulphite Medium** (Wilson and Blair, modified by Read) have also been recommended recently.

Media for Diphtheria Bacilli

and Allison and Ayling's media are useful for isolating diphtheria. Selective antiseptic potassium tellurite solutions should always be freshly prepared from the salt, which is kept dry in the refrigerator.

Johnstone and Zinnemann's Medium.

Low temperature meat extract. 750 to 1,000 grms. of minced meat are extracted with 1,000 c.c. tap-water kept at 48° C. for one hour. The fluid is then squeezed out through lint, left in the ice-chest overnight, and filtered through paper. To 1,000 c.c. filtrate add 20 grms. peptone (Parke, Davis & Co.) and 5 grms. NaCl, warm at 45° C. till solution occurs. Test the reaction by taking 50 c.c. and heating to 80° to 90° C. for 15 minutes, filtering through paper and finding how much N/10 NaOH is needed to add sufficient alkali to bring the whole of the fluid to a 'neutralizing' disk, then filter again through a Chamberland filter, sterilise in the autoclave. Distribute the filtrate in flasks or tubes, several of which should be kept for three days at 30° C. to control sterility. Store the rest in the ice-chest.

Blood-tellurite mixture. Mix five parts of sterile defibrinated rabbit blood or ovalated horse blood with two parts of sterile 1 per cent. solution of potassium tellurite and incubate at 37° C. for one to three days. This mixture keeps indefinitely in the ice-chest; it should not be used until at least three weeks old.

130 c.c. of the meat extract and 130 c.c. of 5 per cent solution of agar in water are thoroughly mixed at a temperature not above 50° C., then 42 c.c. of the blood-tellurite mixture (well shaken immediately beforehand) are added and plates are poured at once.

Hoyle's Medium. A solution containing 1 per cent Lab Lemco, 1 per cent peptone (Difco proteose or Evans), 0.5 per cent NaCl and 2 per cent agar is adjusted to pH 7.8, sterilised in the autoclave and stored in 200 c.c. quantities.

To 200 c.c. of the above agar melted and cooled to 50° C. add 10 c.c. of sterile laked horse blood and 2 c.c. of a 3.5 per cent solution of potassium tellurite mix and pour into plates. (The blood may be laked either (a) by freezing and thawing four times—store frozen, or (b) by adding 0.5 c.c. of autoclaved 10 per cent solution of 'white' saponin to 10 c.c. blood which has been kept at 37° C. for fifteen minutes, mixing gently to avoid frothing, then replacing at 37° C. for fifteen minutes. This keeps in the refrigerator for several months.)

Isolation. For the isolation of the diphtheria bacillus (and diphtheroids) in a pure state, Horgan and Marshall's medium and Allison and Ayling's are useful.

Horgan and Marshall's Medium (modified). To 10 c.c. ordinary 2 per cent agar medium, melted and brought to 50° C. add 2.0 c.c. of a mixture of equal volumes of blood (from ox or horse which has been debarrassed or rendered incoagulable by the addition of oxalate or citrate) and of a 1.5 per cent solution of potassium tellurite in distilled water (a 0.75 per cent solution is preferable for isolation directly from throat swabs being less inhibitory) and pour into plates. The blood tellurite mixture keeps in the ice chest without aseptic precautions for three weeks and is most effective when at least three days old. Parish recommends the addition of 0.5 per cent glucose to the digest broth basis used for the preparation of the agar.

Allison and Ayling's Modification of Douglas's Medium.

(a) 2 per cent nutrient agar (pH 8) with a basis of either (1) Hartley's broth or

(2) meat extract plus 1 per cent peptone (Parke Davis & Co.)

(b) copper sulphate (Analar) 10 per cent solution in distilled water

(c) horse serum 100 c.c.

liquor trypan co. (Allen & Hanburys) 6

potassium tellurite 2 per cent aqueous solution 10

The serum is drawn from horse blood which has been allowed to clot (it should be used fresh and without added chloroform), the liquor trypan co. and potassium tellurite solution are added to the serum and the mixture kept in the refrigerator for twenty-four hours. It is filtered through a Seitz filter into a sterile container; it may then be distributed in amounts of 10 c.c. in sterile bottles and stored in the refrigerator.

To 100 c.c. of the agar melted and cooled to 50° C. are added 10 c.c. of the sterile trypanised serum tellurite mixture and 0.5 c.c. of the copper sulphate solution. After thorough mixing the medium which is transparent is poured into sterile Petri dishes and dried in the incubator; it keeps well.

INOCULANT (MONCKTON'S METHOD). To 100 c.c. sterile Hartley's broth (pH 7.7 to 7.8) add 1.0 c.c. 3.5 per cent potassium tellurite and 0.3 c.c. 10 per cent copper sulphate (Analar) solutions (both in distilled water) and 7.0 c.c. horse blood laked with saponin (as for Hoyle's medium). This mixture does not require to be sterilised further and keeps well. It is distributed in 2.5 c.c. amounts in 1 oz. screw-capped bottles. Inoculate either (a) directly from the swab which should finally be squeezed out thoroughly by pressing it against the glass of the inside of the bottle above the level of the fluid or (b) by adding a large loopful of a suspension of a whole culture on Löffler's serum (made by emulsifying the culture in the condensation water or in several drops of broth). After 6 hours incubation, plate on ordinary blood agar and incubate overnight. Practically pure cultures of diphtheria or diphtheroid bacilli are got in this way.

Selective Antiseptics

The following selective antiseptics are useful for the isolation of the organisms indicated from mixtures with certain common contaminants. The effective concentrations vary somewhat according to the composition of the media.

Cresol violet. Concentrations of 1 in 500 000 to 1 000 000 in agar or 5 per cent blood agar allow streptococci to grow while inhibiting Gram-positive sporing bacilli common in air and dust and also staphylococci. 1 in 100 000 permits growth of most Gram-negative bacilli while killing Gram-positive organisms.

Dispersol L.N. A 5 per cent aqueous solution of this product (1 C.I.) is prepared (the dry powder tends to cause sneezing); it keeps indefinitely. A concentration of 1 in 500 in agar medium (0.4 c.c. of the solution to 10 c.c. agar) permits growth of staphylococci and Gram-negative bacilli but inhibits spread of *B. proteus*. To 10 per cent blood agar (e.g. made with oxalated horse blood) add 0.65 c.c. of the solution per 10 c.c. medium.

Blood not over a week old should be used to avoid haemolysis. In frothing add the re

Silver nitrate 1

also Gram-positive sporing aerobes.

Thallium acetate 1 in 1,000 in agar inhibits *B. pyocyaneus* and *B. proteus*, but allows staphylococci and streptococci to grow; it is useful also for isolating pleuropneumonia-like organisms (Edward)

MEDIA USED FOR THE OBSERVATION OF CHEMICAL ACTIVITIES OF BACTERIA

Fermentation of Sugars, etc. The capacity of certain species of bacteria to ferment

tive processes as an aid to the identification of bacterial species, depend on two kinds of changes, namely (a) the formation of acids, and (b) the evolution of gases (chiefly carbon dioxide, hydrogen, and methane). Generally speaking, these tests are reliable, and the methods to be pursued are simple. For the estimation of other products, alcohols, etc., and the identification of the acids special chemical procedures may be necessary. In addition to such gases as those named some organisms give rise to sulphuretted hydrogen by breaking down the protein.

Besides sugars, closely allied bodies, alcohols and glycosides (which are combinations of a sugar with other substances) may also be acted on by bacteria. It is essential to

substances are commonly employed

Monosaccharides Pentoses—arabinose, xylose, and rhamnose. *Hexoses*—glucose (dextrose), fructose (levulose), mannose, and galactose.

Disaccharides Lactose, saccharose, maltose, trehalose

Trisaccharides Raffinose

Polysaccharides Starch, inulin, dextrin, glycogen, cellulose.

Alcohols Glycerol, erythritol, adonitol, mannitol, dulcitol, sorbitol, inositol

Glycosides Salicin, coniferin, aesculin

In testing the effect of an organism on a given sugar it is essential that this sugar alone be present, the basis of the medium ought therefore to be either peptone solution or casein digest broth (p. 738). In the case of organisms requiring serum for their growth Hiss's serum water medium is used, or peptone water (with or without agar) to which sterile serum has been added. The sugar or other substance is added in the proportion of from 0.5 to 1 per cent. If the sugar is dissolved in the medium and this then sterilised, there is danger that the sugar may be decomposed, in consequence of its being heated in the presence of substances such as alkalis. Therefore it is preferable that the addition should be made in the form of a sterile 10 per cent solution in water—sterilisation being effected in the Koch or by filtration. In any case, the completed medium should not be heated above 100° C. In all tests in which sugars are used, a control uninoculated tube ought to be incubated along with the bacterial cultures, as changes in reaction sometimes occur spontaneously in media containing unstable sugars. Tests in which sugars are used are

DEVELOPMENT OF AN

is formed for some days or even several weeks tested for, by placing the fluid medium

(*infra*) of one part of serum (e.g. ox's) and various of the in the a con-

steam steriliser, the same precautions being

taining sugars. They have been extensively used in studying the fermentative properties of streptococci and pneumococci, etc. As controls, cultures of the organisms should be made in the same specimen of serum water medium from which, however, the sugar has been omitted.

Serum-peptone-water media Peptone water, with or without agar, to which 5 per cent. serum has been added, may be used. The use of unheated serum in such media is a source of fallacy, e.g. horse serum contains amylase and maltase, however, these enzymes are destroyed in one hour at 65° C.

Serum water-starch medium This medium should be used within several weeks of preparation, as the starch gradually hydrolyses on standing. 3 per cent of soluble starch is added to sterile distilled water in a screw-capped bottle, the cap is screwed on and the bottle shaken vigorously, then it is placed in a water-bath, brought to the boil and kept boiling for five minutes shaking being repeated several times, so as to ensure a uniform solution. When cool add, with aseptic precautions, 5 per cent of this solution to the serum water medium, i.e. to 3 c.c. add 0.15 c.c. (5 drops from a Pasteur pipette).

INDICATORS

Litmus. To any of the ordinary media litmus may be added. During the development of an acid reaction the colour changes to pink, and may subsequently be discharged. The disadvantage of litmus is that the colour change with it is not very sharp. The litmus is added before sterilisation, as a strong watery solution in sufficient quantity to give the medium a distinctly bluish tint. Litmus solution is made as follows. Solid commercial litmus is digested with pure spirit at 30° C. till on adding fresh alcohol the latter becomes only of a light violet colour. A saturated solution of the residue is then made in distilled water and allowed to stand until the sediment settles or it may be centrifuged. When this is diluted with a little distilled water it is of a violet colour which further dilution turns to a pure blue. To such a blue solution very weak sulphuric acid (made by adding two drops of dilute sulphuric acid to 200 c.c. water) is added till the blue colour is turned to a wine-red. Then the saturated solution of the dye is added till the blue colour returns.¹

Neutral red. This dye was introduced by Grunbaum and Hume as an aid in determining the presence or absence of members of the *B. coli* group, especially in the examination of water. The media found most suitable are agar or broth containing 0.5 per cent of the sugar to be tested, to which 0.5 per cent of a 1 per cent watery solution of neutral red is added. The alkaline medium is of a yellowish brown colour which in the presence of acid passes into a deep rose red. Sometimes in cultures there occurs subsequently a change to a fluorescent green, caused apparently by a change in the composition of the dye as the fluorescence is not discharged by addition of alkali.

Andrade's indicator. An aqueous 0.5 per cent solution of acid fuchsin is decolorised by the addition of N/1 NaOH. About 16 c.c. are required for 100 c.c. of the dye solution. If the mixture is still red after standing for three hours 1 c.c. further of the caustic soda solution should be added, 1 per cent of this mixture is added to media. Media with a pH of 7.2 are red when hot but faintly yellow when cold. Acid formation restores the red colour.

Bromocresol purple. The sulphone phthalein compound bromocresol purple is a useful indicator. It has a purple colour in neutral or alkaline solution which becomes yellow when the reaction is very slightly acid, hence it is a delicate indicator for detecting acid production. 1 c.c. of a 1.6 per cent alcoholic solution is added to 1,000 c.c. of medium.

Gas Formation is observed by the following methods.

Durham's tube (Fig. 209, a). A small test-tube is inverted and slipped down into the empty culture tube, which is then plugged and sterilised in the autoclave. The medium which has been previously sterilised, is tubed with aseptic precautions and the tubes are finally heated in the Koch for fifteen minutes on each of three successive days. The air remaining in the smaller tube is thereby expelled. The tube is then inoculated with the organism to be tested. Any gas developed collects in the upper part of the inner tube. As some sugars are expensive, it is well to arrange the Durham apparatus with small culture tubes, as with these a satisfactory result can be obtained with only 1 c.c. of

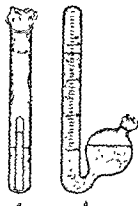


FIG. 209 Tubes demonstrating gas formation by bacteria.

a Durham's fermentation tube.
b South's fermentation tube.

¹ Johnson and Sons, Hendon, London, supply a purified preparation of litmus.

medium (Tubes of medium containing the various sugars etc., may be distinguished by using cotton-wool stoppers dyed different colours)

Smith's fermentation tube (Fig 209, b) This consists of a tube of the form shown, and the figure also indicates the extent to which the bulb ought to be filled, the closed limb, of course, being full. It is inoculated in the bend with the gas-forming organism,

solution of caustic soda, and presence of hydrogen by ignition (see under *B. coli*)

The pH and reaction of cultures. In certain cases it is necessary to ascertain the final pH reached when an organism has grown in fluid medium containing a sugar. For this purpose the initial pH is adjusted and the medium inoculated, then after the culture has been kept for a standard time the pH can be ascertained with sufficient accuracy for most purposes by testing portions with a universal indicator mixture or with a series of indicators which have overlapping ranges. Where only small amounts of fluid are available the B D H 'Capillator' may be used.

The titratable acidity may also be estimated in the usual way by taking a measured volume of culture and, with phenol-phthalein as indicator, finding how much N/10 NaOH is required to restore the reaction to that of the original medium.

Indole Production. The formation of indole from protein by an organism sometimes constitutes an important specific characteristic. To observe indole production the culture is grown, preferably at 37° C., in a fluid medium containing peptone. The latter may either be peptone water or CCY broth made with $\frac{1}{4}$ of the usual amount of yeast—the tryptophane being the component from which indole is formed. Any medium containing sugars must be avoided, as their presence may inhibit the production of indole.

The amount of indole produced by bacteria seems to depend on certain unknown qualities of the peptone. It is well, therefore, to use a specimen which yields a good reaction with a culture of a known indole-producer, e.g. *B. coli*. In general, a well grown forty-eight hours' culture is used for the test, but an older culture may be necessary, e.g. seven days.

Ehrlich's rosindole reaction. Bohme showed that for ease of application and delicacy the reaction possesses great advantages. It depends on the fact that paradimethylaminobenzaldehyde unites with indole to form a coloured rosindole body, especially in presence of an oxidising substance such as potassium persulphate ($K_2S_2O_8$). Two solutions are required

- | | |
|-----------------------------------|---------------------------|
| (1) paradimethylaminobenzaldehyde | 4 grms |
| absolute alcohol | 380 c.c. |
| concentrated hydrochloric acid | 80 " |
| (2) potassium persulphate | saturated watery solution |

To 5 c.c. of a culture of the organism in a suitable fluid medium add 1 c.c. of (1) and then 1 c.c. of (2), and shake well, if indole be present a rose-red colour will appear in $\frac{1}{2}$ to 5 minutes. Sometimes the rose colour appears on the addition of solution (1), and the addition of a special oxidising agent is unnecessary. The rosindole compound can be separated from the culture by shaking the latter with amyl alcohol, and this should be done in cases of a doubtful reaction, as sometimes when a faint pink colour appears in

persulphate solution and then with a few drops of the amyl alcohol. The surface of the culture may be placed in the tube and the plug by the plug.

which in the presence of indole. For the quantitative estimation of indole, Happold and Hogg (1909) show: 10 c.c. of culture acidified with one drop of concentrated HCl are extracted with two successive 10 c.c. amounts of light petroleum (B.P. 40 to 60° C.). A third extraction may be made to ensure complete removal of indole. The pooled extracts are washed once with water. The light petroleum is then extracted with successive 5 c.c. amounts of 1% aqueous solution of sodium hydroxide. The alkali solution is collected in the filter and the reagent should be prepared in the same way. added 1 c.c. at a time. A standard solution of indole is used.

medium as the culture and treated similarly. The rosindole from the culture is then compared with that from the standard in Nessler tubes. Milkyness in the solutions is removed by immersing both tubes in boiling water for the same time, a few minutes being sufficient.

Guedz's test. A strip of filter-paper which has been dipped in warm saturated watery oxalic acid solution and allowed to dry is inserted into the culture tube so that it presses against the side and remains near the mouth. The culture is then incubated. The development of a pink tint in the oxalic acid crystals on the paper indicates the formation of indole. This test is stated to be highly specific for indole.

Sulphuretted Hydrogen Production. Strips of filter-paper about 0.5 by 5 cm soaked in saturated lead acetate solution are autoclaved in a plugged tube and dried at 120° C. A broth or agar culture is inoculated with the organism to be tested and a strip of the paper is inserted into the mouth of the test-tube so that nearly one-half projects beneath the cotton-wool plug. The culture is incubated and examined daily for darkening of the paper, which indicates H_2S . It is important to use in the medium peptone which yields a positive result with a known producer of H_2S such as *B. coli*. Bismuth ammonium citrate incorporated in a suitable medium is stated to be a very delicate reagent for H_2S (Hunter *et al*).

Koser's Medium for Citrate-utilisation. Sodium chloride 5.0 grms, magnesium sulphate 0.2 gm., ammonium dihydrogen phosphate 1.0 gm., dipotassium hydrogen phosphate 1.0 gm., all dissolved in 1 litre distilled water. To the solution add 2.0 grms. citric acid and bring the pH to 6.8 with N/1 NaOH. Tube in 5 c.c. amounts and autoclave at 120° C for 10 minutes. This alternative formula (vide p. 315) is recommended in *Rep. Pub. Hlth. Med. Subj.*, No. 71, 1939 (Ministry of Health).

THE MAKING OF CULTURES BY USE OF THE ORDINARY CULTURE MEDIA

Cultures may be made in order to grow organisms either from material suspected to

organisms or when these are required in large quantity.

Manipulations involving the transference of small quantities of bacteria to a medium, as in the inoculation of tubes are effected by platinum wires (Nos. 26 or 28 Imperial

Standard wire gauge—corresponding to 0.457 and 0.381 mm. thickness) fixed in glass rods 8 in. long or in aluminium holders. A substitute for platinum wire is 'resistance wire', No. 27 I.S.W.G. mounted in an aluminium handle. Every worker should have two wires. One is 2½ in. long, and has a loop turned upon it (Fig. 210, a), this is referred to as the 'loop' and is used for many purposes—

taking a 'loopful' is a phrase constantly used. The other wire (Fig. 210, b) ought to be 4½ in. long and straight, it is used for making stab cultures. A platinum-iridium spud is also very useful for making scrapings from organs and for disintegrating felled bacterial cultures, in such manipulations the ordinary wire bends too easily. The spud consists of a piece of platinum-iridium about 3.75 cm. long, 1.3 mm. broad and of sufficient thickness to give it a firm consistence. Its distal end is expanded into a diamond shape and its proximal is screwed into an aluminium rod.

If a platinum wire heavily charged with bacteria be sterilised in a Bunsen flame 'sparking' may occur and un-killed bacteria thus fall on the bench. In working with

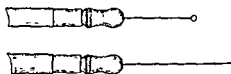


FIG. 210. Platinum wires in aluminium handles (Reduced in size.)

- a. Platinum loop.
- b. Straight wire for ordinary puncture inoculations.

sublimate instead of being heated

1 Pseudocolonies are an occasional fallacy, these consist of local precipitates from the medium which resemble colonies to the naked eye and which may yield a subculture on transfer to fresh medium.

Cultures on a solid medium are referred to as (1) 'puncture' or 'stab' cultures when made on medium solidified in the upright position; such cultures, *e.g.* in agar, are suitable for maintaining organisms alive for long periods, since they dry up less quickly than stroke cultures. (2) Cultures made by inoculating the surface of *sloped medium* are called 'stroke', 'slope', or 'slant' cultures. Similar stroke cultures may be made on

To make a subculture on an agar slope from another culture. The two tubes are held between the thumb and first two fingers of the left hand, in a slanting position, towards their lower ends, with the *sloped surfaces upward*, the mouths of the tubes being at least an inch apart. Then with the right hand rotate the cotton-wool stoppers of both tubes,

hand remove the stopper of the tube from which the inoculum is to be taken, and retain it there; flame the mouth of the tube. Pass the needle into the tube and touch the medium to ensure that the wire is cool (if the wire is too hot it will be seen to melt the

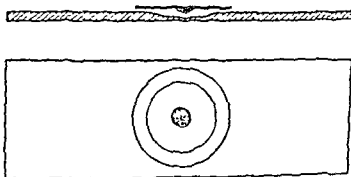


FIG. 211.

Hollow-ground slide for hanging drop culture, shown in section and plan

medium, one with the wire, the points of tube, insert (avoid cutting into the medium) Withdraw the wire, *pass the* mouths of the tubes and replace the stoppers in their respective tubes; then *sterilise the wire in the flame before laying it down* (Label the fresh culture at once with the name of the organism and the date)

A simple method of obtaining large amounts of surface growth on solid medium has been described by Epstein and Chain

To make a stab culture Use the straight wire charged with culture and pass it into the centre of the medium, taking care to withdraw it in the same track, so as to avoid

of the contents being incorporated with the suspension is produced When large quantities of culture are required, it is often advisable to use bigger inocula This is the case also when delicate organisms are being subcultured When the inoc

Hanging-drop Cultures.
the microscope hanging-dro

the microscope hanging-dro

middle of the sterile cover-glass. If the organism is growing on a solid medium, a loopful of sterile broth is placed on the cover-glass, and a very small quantity of the culture (picked up with a wire) is rubbed up in the broth. The edge of the hollow in the slide is then painted with vaseline, and the slide, held with the hollow surface downwards, is lowered on to the cover-glass, to which it of course adheres. The slide with the cover attached is then quickly turned right side up. The method of microscopic examination is described later.

To make a hanging-drop culture with a solid medium a drop of melted gelatin or agar is substituted for broth and the surface inoculated after solidification.

Hanging-drop culture of moulds. An india-rubber washer $\frac{3}{8}$ in. high, about $\frac{7}{8}$ in. internal diameter, and of a size to lie completely on a slide is luted on with lanolin, and a drop of water is placed on the slide within the ring. Then a large drop of culture medium (fluid or melted) inoculated with the mould is placed on a thin sterile slide. The slide with the drop is inverted over the rubber ring, the surface of which has been smeared with lanolin, so that an air-tight cell results.

INOCULATING ROOM OR HOOD. Where there is special liability to contamination from atmospheric dust some form of inoculating room or hood should be used in which to make cultures. In its simplest form this consists of a large oblong box of wood or metal which sits on the bench. It has glass windows on three sides, the fourth side, corresponding with the front of the bench, consists in its upper part of a sloping glass window, while the lower part has two apertures through which the hands and arms can be inserted. These apertures are protected by flaps or sleeves of rubber. A ventilator lightly packed with non-inflammable cotton wool is fitted on the top. Electric light and a Bunsen burner are provided. The inner surface is freed from dust by wiping with a cloth soaked in antiseptic solution and about half to one hour before use the interior is well sprayed with water from a fine spray so as to remove floating dust as far as possible.

Growth of Cultures in Air containing Excess of Carbon Dioxide

The addition of 5 to 10 per cent of CO_2 to the atmosphere improves the growth of many bacteria and is specially advised when solid CCY medium is used. A simple method of securing this excess is to incubate the cultures in cylindrical tinned iron boxes with press-down lids in which CO_2 is generated. For a 6 in. by 8 in. box (about $3\frac{1}{2}$ litres capacity) 5 per cent CO_2 is obtained by inserting along with the cultures an open tube containing 8 c.c. of 25 per cent HCl into which a marble chip weighing about 0.7 gm.

momentarily to enable cultures to be taken out or inserted without much change occurring in its atmosphere. When a larger proportion of CO_2 is required, cultures may be placed in an anaerobic jar, the air reduced to the necessary extent by a pump and replaced by CO_2 from a cylinder, etc.

THE SEPARATION OF ORGANISMS IN CULTURES

Plate Cultures. The general principle underlying methods of separation by plate cultures is the distribution of the bacteria in or on a solid medium, so that the colonies formed by the individual organisms (or small aggregates of these) are sufficiently far apart to allow their being examined separately. For the purpose circular shallow glass capsules, each fitted with an overlapping cover (usually of glass) are generally used, these are known as Petri's plates or dishes (Fig. 212). The medium (15 c.c. for a 4-in. plate), after being melted, is poured into a sterile plate and allowed to solidify, so as to form a thin layer. In this way the colonies which afterwards grow are readily accessible. (It is advisable to cool the agar to about 50°C . before pouring, so as to minimize formation of condensation-water. The plate should be gently rocked immediately on pouring to ensure even distribution of the agar, and in cool weather the plate may be slightly warmed beforehand to obviate the agar setting before it forms a uniform layer.) In one method the material containing the bacteria is smeared over the surface of the medium after it has solidified in the plate—'method of successive strokes'. In another method the organisms are mixed with the medium when in the melted state and the mixture is then poured into the plate and allowed to solidify—'dilution method'.



FIG. 212 Petri's plate
(Cover shown partially raised)

The method of successive strokes is convenient and is with-
 cedure have it when it which it is placed in the incubator for half an hour, or drying may be effected after
 by warming the open plate melt the med can be sterili are on a swc
 surface of the medium, always with it. In this way the o strokes they may be deposi The method of spreading de plates should always be incu the surface by condensation are kept with the medium as liquefaction often occurs from the growth of organisms Sometimes it is advisable to smear several plates consecutively with the

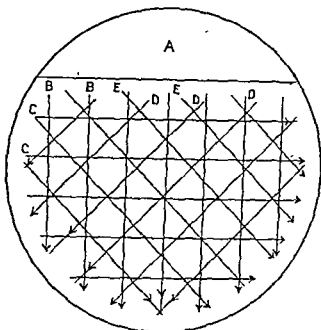


FIG. 213 Diagram of method of inoculating plate from faeces. (After Medical Research Council's Special Report No. 51)

same portion of the swab. If the material to be examined is fluid, e.g. an emulsion of faeces, a usual method is to place a loopful on the surface of the medium, and then, with a sterile glass rod bent at a right angle, to smear found, on microscopic examination, to be very to dilute with sterile broth before making the which appear on the plates can be examined with a hand-lens, magnifying about 6 diameters. In some cases examination under a low power of the microscope is an advantage, the plate in the inverted position can be put on the stage of the microscope for this purpose. For the culture of special organisms, as afterwards detailed, other media are used according to the growth requirements of the organisms.

When making enteric and dysentery isolated colonies

loopful of fluid faeces or of faecal emulsion is spread on the medium at one place on the plate in the area A, then, without recharging the needle successive series of parallel strokes are made in different directions (e.g. a series in the direction indicated by the lines B, a second series in the direction shown by the lines C, and so on)—the flat part of the loop being throughout kept in contact with the medium. Plates with a diameter of 4 in. are useful. As the layer of medium must not be too thin, about 15 c.c. is sufficient.

The principle of successive strokes may be applied also to agar in tubes, although not so satisfactorily. Several agar tubes are taken, a platinum loop is charged with the

material to be examined, and in each tube several vertical strokes are made from below upwards on the surface of the agar, one tube after the other being used without recharging the needle. The tubes after inoculation should be kept in the upright position, so that the water of condensation will not run over the surface.

Dilution method In this method the bacteria are added to the medium when liquid and mixed by rolling the tube between the palms of the hands (to avoid shaking, which causes air bubbles); the inoculated medium is then poured out into a plate and allowed to solidify. As in this case the organisms are distributed throughout the medium some

plate being prepared for each. If gelatin is used, the medium in tubes is melted and kept in water at about 28° C. If agar is used, the medium is melted by boiling in a water-bath and then cooled in a water-bath to about 45° C. at which temperature the inoculations are made. The following are the details.

The contents of three tubes, marked (with a grease pencil) *a*, *b*, *c*, are liquefied as above

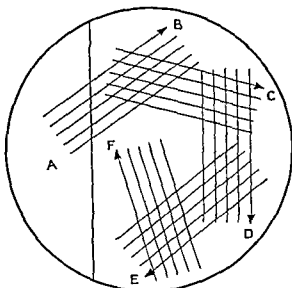


FIG. 211 Diagram of alternative method of inoculating plate from feces, etc.

described. Inoculate *a* with the bacterial mixture. The amount of the latter to be taken

loops of medium from *a* to *b*. Mix *b* and transfer five loops to *c* and mix. The plugs of the tubes are in each case replaced and the tubes returned to the water-bath. The con-

place

For accurate work it will be found convenient to carry out the dilutions in definite

to the third and so on. There is thus effected a twenty-fold dilution in each successive tube. Finally, a definite amount, say 0.05 c.c., is transferred from each tube to a tube of melted medium, the medium being afterwards plated and the colonies counted when growth occurs. (One pipette will serve for the series if the highest dilution is plated first

impin... that bearing the culture... occupied by the puncture mark is... (The method has been modified by Gardner)

The method of Topley, Harnard and Wilson is as follows. A loopful of a young broth culture of the organism (*e.g.* after six hours incubation) is used to inoculate a tube of 10 per cent gelatin in 1 per cent peptone water previously melted and cooled to 37° C. This culture is kept for two hours in the incubator, then a loopful (external diameter of loop, 3 mm) is placed on a sterile circular quartz cover slip of 12 mm diameter which is inverted on a sterile slide so as to yield a thin film free from bubbles and not ending at the edge. By examination with dark-ground illumination under a dry $\frac{1}{4}$ -in. objective, a well-isolated organism towards the centre of the cover slip is selected. Under the low power a droplet of mercury is picked up with a rough, rusty steel needle, placed on the cover-slip, and then pushed along with a needle until the selected organism is covered. The preparation is then exposed to a suitable source of ultra violet light for a time sufficient to kill all the other bacteria that under the mercury being protected, to prevent the action of oblique rays the exposure is made through a tube of about 24 mm diameter. By way of control similar preparations without drops of mercury are exposed to the light in the same way. These should prove sterile on subsequent incubation at 25° C., whereas the screened organism if viable will have formed a small colony. A subculture is then made from the latter.

Another method consists in the use of a micro-manipulator such as that of Schouten.¹ (For details, reference must be made to the original papers.)

THE CULTURE OF ANAEROBIC ORGANISMS

All ordinary media contain traces of free oxygen and absorb more from the air on standing. Therefore media intended for the growth of anaerobes should always be heated at 100° C. for ten minutes immediately before inoculation in order to remove the oxygen. Reabsorption of oxygen can be minimised in the case of shake cultures in solid medium by using deep tubes. In the case of anaerobes it is especially important that the medium should be free from copper. It is advantageous if the media contain a reducing agent which does not interfere with bacterial growth. Such an agent takes up any oxygen which may already be in the medium and prevents further accumulation. Glucose (1 per cent) or sodium formate (1 per cent) was originally used for this purpose but more effective reagents are now employed (*vide infra*).

Fluid Media. In the case of broth Noguchi's tubes were long employed. These are narrow tubes (8 in. by $\frac{1}{4}$ in.) which are half filled with medium and then heated in the Koch for thirty minutes or in boiling water for ten minutes. Melted vaseline (previously sterilised at 150° C.) is then poured on the surface of the medium and the tubes are cooled quickly. Inoculation is effected by means of a capillary pipette after melting the vaseline, which acts as a seal excluding the air. This method is not suitable for the culture of organisms which produce much gas as the vaseline plug is forced out of the tube.

Anaerobes will grow in fluid media without excluding the air, however, if constituents are present which lead to the disappearance of oxygen and exert a reducing action, this is effected by having portions of animal or vegetable tissue present. Sometimes fresh tissues which contain catalase have been employed, *e.g.* portions of kidney. In many cases heated tissues act well, as in Robertson's cooked meat medium. The addition to the medium of reducing agents such as metallic iron, thioglycolic acid, or ascorbic acid is also very effective. It is advantageous to add to such fluid media also a small amount of agar *e.g.* one tenth volume of ordinary agar medium.

Cooked meat medium. According to Martin and Lepper the disappearance of oxygen is chiefly due to auto oxidation of unsaturated fatty acids of the lipins contained in the meat, the reaction being catalysed by the muscle haematin. Heart muscle is preferable because it contains about twice as much lipins as other flesh. In addition the small amount of glutathione which is present and the fixed sulphydryl groupings of the muscle proteins produce a negative oxidation-reduction potential. Further, the solid fragments when packed together minimise the entrance of oxygen into the medium. Thus even asbestos wool has been found effective in making a fluid medium suitable for growth of anaerobes.

Metallic iron. The tubes of medium are heated in a boiling water bath for ten minutes and quickly cooled without shaking. Then to each a 3 mm. by 25 mm. strip of

¹ Supplied by Dr S. L. Schouten, Hygienic Laboratory, The University Utrecht

'sheet iron', gauge No 26, which has been stamped out, making red hot in a Bunsen flame.

Thioglycollic acid 0.02 to 0.05 c.c.

tenth of its volume of ordinary water, and the pH adjusted to 7.4 with NaOH solution. The mixture is sterilised by autoclaving or in the Koch; it keeps at room temperature for some weeks. Before use it should be heated and the details of Brewer's thioglycollic medium.

Ascorbic acid Most anaerobes will grow on ascorbic acid. Solutions may be sterilised by autoclaving. The media and solutions are usually sterile. A 'knife-point' of the powder as can be taken up by a 2 mm of medium at the time of inoculation, or 0.5 c.c. of a 5 per cent solution of the tablet may be used.

Surface Growths on Solid Media are obtained by removal of oxygen in several different ways (1) displacement by an inert gas, usually hydrogen, but nitrogen may be used; (2) combustion combined with displacement by hydrogen; (3) absorption with sodium

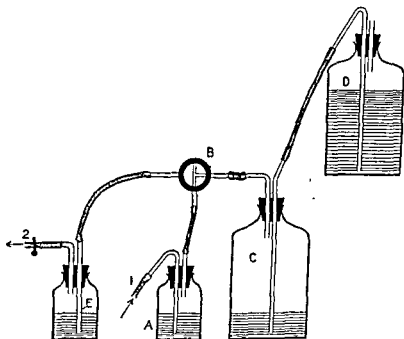


FIG. 215. Arrangement of bottles for reducing pressure in hydrogen supply from cylinder. (After Mackie and McCartney.)

pyrogallate, (4) absorption by bacterial growth, (5) addition to the medium of a reducing agent such as ascorbic acid.

Hydrogen The gas is generated in a large Kipp's apparatus from pure 25 per cent sulphuric acid and pure zinc. It is passed through three wash-bottles. In the first is placed a solution of lead acetate (1 in 10 of water) to remove any traces of sulphuretted hydrogen. In the second is placed a 1 in 10 solution of silver nitrate to remove arseniuretted hydrogen which may be present if the zinc is not quite pure. In the third is a 10 per cent solution of pyrogallic acid in caustic potash solution (1 in 10) to remove traces of oxygen. The tube leading from the last bottle to the vessel containing the medium ought to contain a small plug of cotton wool to filter any organisms out of the hydrogen, and the tube with the plug should be sterilised by hot air before use.

Commercial hydrogen as sold in cylinders may be used, if it is necessary, however, to reduce the pressure, and this can be done as follows. The hydrogen from the cylinder is passed by tube (1), not too rapidly, through the three-way stop-cock B to a jar C of roughly 1 litre. The jar C is furnished with an indiarubber stopper and tubing as shown. The hydrogen entering C forces the water into a similar jar D which is supported 4 ft above C. When sufficient gas has entered C the supply valve from the cylinder is closed. The stop-cock B is then turned, so that the hydrogen passes from C through the wash-bottle E, and passes by tube (2) to the anaerobic jar, the flow being adjusted so as to produce a not too rapid stream of bubbles. As the gas escapes from C it is replaced by

water from D. All stoppers must be air-tight, this can be secured by means of sealing wax. All the rubber tubing must be of the thick-walled 'pressure' type.

An alternative method is to attach a *reducing valve* to the gas cylinder, which will deliver the hydrogen at a constant pressure e.g. of 2 or 3 lb. per sq. in. as indicated by a gauge connected to the delivery tube. The flow of hydrogen into the anaerobic jar can be observed by interposing a small wash-bottle between the valve and the jar, similar to that shown in Fig. 215.

Hydrogen displacement plus combustion—*McIntosh and Fildes' anaerobic jar*. These authors have designed an apparatus in which tubes, etc. may be incubated under anaerobic conditions, the oxygen being absorbed by means of spongy palladium. A glass jar is employed furnished with a metal lid which can be clamped down. The lid is fitted with a tube and valves so that hydrogen may be admitted into the jar. The palladium asbestos is fixed on an insulated spool and surrounded by a piece of resistance wire which is connected to two electric terminals on the outside of the lid. The jar is closed and hydrogen passed through it for half a minute. Then the valves are closed and the terminals are attached to the electric supply, which must be reduced by suitable resistances. As a result of the consumption of oxygen by the hot palladium asbestos the pressure within the jar falls, and after the current has been passing for ten minutes more hydrogen may be added at very low pressure, the current should then be allowed to pass for half

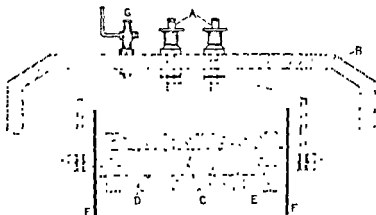


FIG. 216. Top of anaerobic jar (Fildes and McIntosh).

A. Electric terminals. B. Section of metal top of jar. C. Coil of wire around insulator.
D. Asbestos. E. Gauze cover. F. Mica plates. G. Gas inlet.

an hour. Along with the cultures an open test tube containing an *indicator* should be placed in the jar. This consists of 1 per cent. glucose broth of pH 8.5 to 9 tinted strongly with methylene blue solution and preserved from mould by the addition of 0.01 per cent. phenyl mercuric nitrate or merthiolate or by a small piece of thymol. This indicator, when in the jar, should remain colourless except for a slight tinge of blue at the top which slowly disappears during the passing of the current. To secure very thorough anaerobiosis current may be passed at intervals during the period of incubation. It is essential that the lid of the jar should be air-tight. This can be tested by placing a few drops of ether in the jar, fixing on the lid, and plunging the vessel in hot water; any leak can thereby be detected. To avoid danger from accidental explosion while hydrogen is being introduced, glass jars should be covered with a cloth and placed in a wooden box. Metal jars are obtainable also.

To prevent excessive spreading of colonies on the surface of the agar through moisture, an open Petri dish containing dry CaCl_2 should be placed in the jar.

Hudson's modification. This consists of a metal tin with a press-on lid in the centre of which is soldered a small gas-tight brass tap, opposite this on the under surface of the lid there is a folded strip of brass which holds a brass wire-gauze capsule containing palladised asbestos. On the side of the tin towards the top a hole is pierced into which a bent metal tube is soldered, this is connected by a short piece of indiarubber tubing with a small glass quill tube sealed at the lower end and filled with methylene blue indicator (*vide supra*). For use under anaerobic conditions cultures are placed in the tin, plasticine is spread with a rag moistened with spirit round the edge of the top and the capsule is heated in a Bunsen flame. Then the lid is at once pressed down, the indiarubber tube from the hydrogen supply is attached to the nozzle of the open stopcock and the gas is turned on, the plasticine is moulded over the edge of the lid. After

fifteen to twenty minutes, when the tin is quite cold, the tap is closed and the tin disconnected and placed at 37° C. When anaerobic conditions are satisfactory the indicator becomes colourless in two to three hours.

Chromium sulphuric acid

hydrogen. The CrSO_4 forms reducing $\text{Cr}_2(\text{SO}_4)_3$ and water. placed, first a 15 per cent metal powder (100 c.c. of the acid solution and 5 grms. of the metal per litre capacity of the jar); next introduce into the jar cultures on a glass or earthenware stand and also a tube of methylene blue indicator solution (*vide supra*). Place the lid on the jar (making the junction gas-tight with plasticine or a grease-wax mixture), leaving the stop-cock the jar near a (Mueller).

container of in 145 c.c. been used

consists of a tube of thick glass measuring about 8½ in. by 1 in., with the lower end constricted, so that the culture tube does not reach the foot. The Buchner's tube is provided with a tightly fitting indiarubber stopper. Solid caustic soda (three pieces each of about the size of a pea or 2 grms. of pellets) is placed in the bottom of the tube and covered with several layers of filter-paper, about 15 c.c. 10 per cent solution of pyrogalllic acid are added, then the inoculated culture tube is quickly introduced and the tube is closed with the stopper.

Another method consists in inoculating an agar slope in a thick-walled test-tube, cutting off the free end of the absorbent cotton-wool stopper till about 2 cm. remains, pushing the stopper into the tube for 2 cm. and filling the space with NaOH pellets, then pouring in 4 to 5 c.c. 10 per cent. pyrogalllic acid solution. The tube is finally closed with a well-fitting indiarubber stopper, inverted so that water of condensation does not run over the agar surface, and incubated in this position.

Bacterial growth. This method depends on the fact that certain organisms when growing in a confined space actively remove the oxygen in the contained air. A ring of aluminium one-ninth of an inch thick and with an aperture of 3 in. is made to fit closely the hole in the Petri plate. Melted agar is poured into the space in the ring and,

In order to avoid contamination from creeping insects, the plate through condensation should not be removed.

Use of ascorbic acid,

acid (*vide supra*) is poured on the surface is inoculated and then covered with a disk of cellophane (which must not exceed the internal diameter of the plate), this is smoothed with a sterile glass spreader to get rid of any air bubbles. Finally 12 c.c. of melted ascorbic acid agar is poured on top. When the medium is solid the plate is incubated, and characteristic colonies of surface growth develop underneath the cellophane. The disk and overlying agar can readily be stripped off, but as this destroys the appearance of the colonies, their features must be noted beforehand. Thioglycolic acid (0.05 per cent) may be used similarly.

used

50° definite blue tint. After three hours at 37° C. the medium should be colourless except for a well-defined blue zone at the top 4 mm. deep, by the third day the blue zone should not extend below 9 mm. Accordingly a layer of ascorbic acid agar at least 12 mm. thick will permit growth of anaerobes in its depths.

It is often more satisfactory to obtain anaerobes, and then cultures with may be made in to 45° C. The

is incubated as usual. To make subcultures from separate colonies in such a shake culture a 'Pasteur pipette' (made by drawing out a piece of quill tube to a capillary at one end and plugging the other end with cotton wool) previously sterilised by heat, is passed down to the colony and some of the growth is sucked up into it. This method, however,

ord-
rom
ring
A

mixed culture in which the more resistant survives. Again, in surface growths, certain organisms, e.g. *B. tetani*, tend to spread out farther than others and so to be found pure at the margins. By means of animal inoculation pathogenic members may be recovered from mixtures with non-pathogens, or where several pathogenic types are present, the wanted organisms may be permitted to flourish alone in the body of an animal passively immunised beforehand by an injection of anti-serum to the other species in the mixture.

INCUBATION AND STORING OF CULTURES

For the purpose of maintaining a uniform temperature of 37° C at which most pathogenic bacteria grow best, incubators are used. These vary in the details of their structure, but all consist of a chamber provided with a source of heat regulated by a thermostat. Patterns in which gas or oil is used have double walls between which fluid (usually water) is placed (Fig 217). Electrically heated incubators, without water (anhydric) are very convenient. Where large quantities of cultures are dealt with, a warm room is advantageous: the temperature of this is regulated similarly to that of an incubator, but to prevent undue cooling on entering the room, it is provided with double doors the space between which acts as an air-lock, so that the outer door is closed before the inner is opened and vice versa.

It is necessary to prevent excessive evaporation from the surface of cultures placed within incubators, otherwise they may quickly dry up. With tubes which will require prolonged incubation the plugs should be pushed a little way into the tube and a few drops of melted paraffin dropped on the top of the wool or the plugs should be covered by disks of paraffin wax which are softened by heat and pressed on to the mouths of the tubes or by indiarubber caps previously sterilised in 1:1,000 corrosive sublimate and then dried. In all cases the cotton-wool plugs ought to be well singed in a flame before covering. Cultures in bottles with screw caps are also protected from evaporation. Another method of preventing evaporation which is useful for the maintenance of stock cultures is to keep them as stab cultures. Incubators are usually maintained also at other temperatures—20° C for cultures on gelatin medium which melts at about 22° C (gelatin cultures may be grown at the laboratory room temperature), 30° C for organisms which grow better below blood heat or which exhibit special characters when growing at the lower temperature, e.g. *B. pestis*.

Generally, for preserving the life of bacteria in cultures the medium should not contain

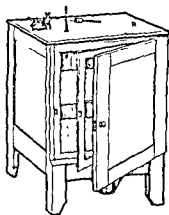


FIG. 217. Hearson's incubator for use at 37° C (form heated by gas).

gonococcus, etc., may be kept alive for two and a half years (McLeod). Cultures in fluid

medium which have been grown at 37° C. for eighteen hours or suspensions in broth of twelve to forty-eight hours' cultures on solid medium are used. A drop of such suspension is placed in a small sterile tube, which is plugged with cotton wool. This tube is then lowered into a wider test-tube, so that it rests on a layer of P_2O_5 , 8 mm deep, which has been covered with a small pad of cotton wool. The mouth of the larger tube is then constricted in the Bunsen flame to aid sealing, and it is connected with an air pump until the drop of culture in the inner tube is dry (a few minutes). The tube is then sealed to maintain a vacuum and stored in the dark at room temperature. To obtain a subculture, emulsify the dry material in a few drops of broth and inoculate a suitable medium. The 'lyophil' process (p 770) is especially valuable for preserving cultures.

FILTRATION OF CULTURES, ETC.

For various purposes it may be necessary to filter all the organisms from fluids in which they are present. This is done especially in preparing media with sterile fluids, such as serum, which cannot be heated, in obtaining the soluble toxic products of bacteria, and also when investigating 'filter-passing' viruses. Filters capable of keeping back

ducc
grain
(French) filters varies and is specified by the maker. L 1-3 grades are of increasing fineness but the last may allow some bacteria to pass. L 5-13 grades are stated to retain all the ordinary bacteria. Doulton (British) filters are similar to the above. There are several other types of filters, differing in character, e.g. the Berkefeld filter made of diatomaceous earth (there are three grades of porosity, designated V, N, and W in diminishing order) and the Mandler (American). In the Seitz type of filter, a disk of asbestos material (Ford's S B grade 'sterilmat') is used as the filtering agent (grade GS is more permeable). 'Two-layer' sintered glass filters are also available for retaining bacteria. For the study of viruses by ultra-filtration, extremely fine-pored filters are obtained by the use of Elford's 'gradocol' collodion membranes (see the section on

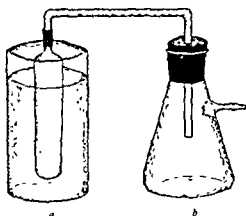


FIG. 218 Chamberland candle and flask arranged for filtration

For ordinary bacteriological work, the most generally convenient filtering apparatus is that in which the fluid is sucked through the porcelain filter or an asbestos disk by exhausting the air in the receptacle means of a water-exhaust pump from the main, all connecting

otherwise solid particles which are ductile, may be time of filtration is prolonged, organisms may grow through the filter. In a practical work involving filtration, it is necessary to note the area of the filtering surface, the amount of filtrate, the pressure at which filtration takes place, and the duration of the process. All the apparatus must, of course, be sterilised before use (vide *infra*).

Filters are arranged in various ways. (a) In an apparatus as shown in Fig. 218, the fluid to be filtered is placed in the cylindrical vessel *a*. Into this a 'candle' or 'bougie', as it is called, is placed with thick rubber connections at the top and bottom. The pump is connected to the bottom of the filter.

When the pump is started, the fluid is sucked through the filter. In a practical work involving filtration, it is necessary to note the area of the filtering surface, the amount of filtrate, the pressure at which filtration takes place, and the duration of the process. All the apparatus must, of course, be sterilised before use (vide *infra*).

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is placed inside the candle, small amounts can be filtered in this way.

(d) The disk of the Seitz type of filter is inserted into a holder, which is fitted into the neck of a filter flask instead of the candle, etc., shown in Fig 210, *a*.¹ It is effective and convenient in use. A fresh disk is employed for each filtration.

Before any one of the above apparatus is used it ought to be washed by passing distilled water through until the filter does not change the pH of the wash-water. Then it should be connected up as far as possible, but loosely, and sterilised in the autoclave or Koch's steriliser. The ends of any important unconnected parts ought to have pieces of cotton wool tied over them. In the Seitz filter, just before use, the disk should be moistened with sterile saline, so as to soften it before it is screwed down tightly in place.

Material to be filtered should be freed from gross particles by centrifuging or

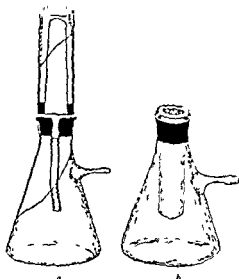


FIG. 210 Other arrangements of filtering candle and flask

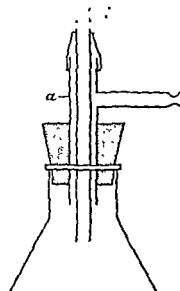


FIG. 220 Fitting for attaching filter to flask. After Blackie and McCartney, *Handbook of Practical Bacteriology*.

be used—*a* is a piece of brass tubing with a side arm, it is fitted into a stopper for a flask of the size required to hold the filtrate. The brass tube is wide enough to accommodate *b*, which is a piece of brass or glass tubing 6 to 7 mm. in external diameter

minute perforations in a filter sufficient to allow bacteria to pass through. It is often advisable to add to the fluid immediately before filtration a suspension of an agar culture of an easily identifiable organism such as *B. prodigiosus*, or a suspension in the same fluid as that filtered, and in similar amount, may be passed through the filter immediately after the main experiment.

After use a candle is sterilised in the autoclave or by soaking in antiseptic solution. Much of the material kept back on the filter can now be removed by forcing water through in a direction opposite to that of the flow of the fluid during filtration, but this is a slow process and to be effective must be continued for some days. Colloidal matter tends, the filter and may be dis- then passing y water, until

the filtrate reacts neutral. Alternatively, the candle, after being dried, may be carefully burned in a muffle furnace till the original colour is regained, there is, however considerable risk of cracking.

Aseptic distribution. In order to avoid the risk of contamination entailed in transferring the filtrate to another flask, the fitting shown in Fig 220 may

¹ A form of this filter in which the holder is made of glass and stainless steel, is supplied by Messrs A. Gallenkamp, 17 Sun Street, London, E.C. 2

and
indie

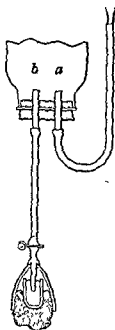


FIG 221 Arrangement for distributing sterile fluid aseptically into a series of containers

stopper is substituted for that with the filter attachment

An indiarubber stopper to fit the flask carries two tubes (*a* and *b*)—tube *a* has an indiarubber tube attached which must be longer than the height of the flask and which at its other end carries a short piece of glass tube plugged with cotton wool (this serves as an inlet for air); a glass nozzle which is protected by a glass hood is attached to tube *b*. The whole apparatus is wrapped in kraft paper and sterilised. It is inserted, with aseptic precautions, in place of the stopper of the flask. The flask is clamped in the inverted position, care being taken to keep the free end of the tube attached to tube *a* above the level of the fluid. This arrangement can be adapted to screw-capped bottles.

Other aspects of filtration are discussed in the section on Viruses.

Clarification is employed when fine particles in a medium are not readily removed by filtration through paper and when the method used for the preparation of media (p 740) is not applicable. Also it is an essential preliminary to filtration where gross particulate matter is present, e.g. in tissue suspensions which are to be investigated for viruses. This may be effected by centrifuging, passing through an F.C.B. grade 'sterilmat', or a 'sintered' glass filter, or a pulp clarifier. The latter may be made by beating up T. B. Ford's 4 per cent. asbestos-cotton filter pulp in one hundred times its weight of water and placing a layer between filter-papers (Whatman No 1 or Green's No 401) in a Buchner's funnel (5 grms. pulp serve for a 5½-in. funnel).

THE DRYING OF SUBSTANCES *in vacuo*

As many substances, for example toxins and antitoxins, with which bacteriology is concerned would be destroyed in drying by heat as is done in ordinary chemical work, it is necessary to remove the water at the ordinary room temperature. This is most quickly effected by drying *in vacuo* in the presence of some substance, such as strong sulphuric acid, calcium chloride, or phosphorus pentoxide, which readily takes up water vapour. The vacuum produced by a water-pump is not suitable here, as in such a vacuum there must always be water vapour present. An air-pump has therefore to be employed.

The *lyophile* process—evaporation from the frozen state *in vacuo*—is of special value for biological products, which are then stored *in vacuo* in the refrigerator (see Flosdorf and Mudd).

REFRIGERATION

Some form of refrigerator which will maintain a temperature of -10° to -15° C is of the greatest value for preserving antisera and other biological products. The presence of 0.35 per cent. cresol in antisera (tetanus antitoxin) which have been kept frozen, does not have any appreciable effect on their antibody content (see Hartley). Deterioration may occur, however, when dilute antisera containing larger amounts of phenolic antiseptics are frozen.

CENTRIFUGES

each of a
hand-drv
or more,

with a speed
centrifuge tubes

should be provided with loosely fitting metal caps or where sterility is essential screw-capped bottles should be used. The centrifuge should start and stop gradually. Centrifuges with speeds up to 15,000 r.p.m. are useful. For the investigation of viruses high speeds are essential, e.g. up to 60,000 r.p.m. in McIntosh and Selbie's apparatus on Henriot and Huguenard's principle. In the centrifuge of Discoe, Pickels and Wyckoff, modified from Svedberg's, the centrifugal force developed is estimated at 180,000 to 225,000 times that of gravity.

MOUNTING BACTERIAL CULTURES AS PERMANENT MUSEUM SPECIMENS

	(Muir) When the
	urating the cotton-
	: up the following
thymol water (saturated in cold)	30 c.c.
glycerol	20 "
potassium acetate	5 grms
gelatin	10 "

Render the mixture acid to litmus with acetic acid, clear with white of egg or serum,

pool plug from the culture, allow
over the surface of the medium

allow

ep their appearance for about
by allowing the culture after
overnight then immersing it in
nitrate 7.5 grms chloral
0 c.c., water 925 c.c.)
(running tap for several
ylated spirit for twenty-
colour should now have
d by placing it inverted
of the agar, and the
well preserved

PREPARATION OF GLASS-WARE

New glass-ware should not be unpacked in the laboratory, as the dust from straw
Test tubes, flasks, etc., should be well washed
the commercial detergents, e.g. $\frac{1}{2}$ to 1 per cent of
ghly rinsed in running water and sterilised. The

well as water
cent phenol red solution to impart a colour

ends glass tends to give off alkali. In
r cent HCl and rinsed
add sufficient 0.04 per
autoclave for thirty

minutes at 120° C. If the glass is not of good quality, it will become yellow. A shows that yield alkali on the first autoclaving.

of the solution will remain the glass. If the first test is negative, as good quality glass may

DISPOSAL OF INFECTED APPARATUS, ETC.

It is essential to prevent infective material from drying and being disseminated in the air. Draughts should be avoided in the rooms where such materials are being examined or handled. Materials should only be left open in the laboratory.

B. tularensis, *Br. melitensis*, etc., infection, these are dealt with in the respective sections.

All contaminated tubes, flasks, plates, hanging-drop cultures, etc., which contain non-sporing bacteria ought to be at once placed in a large dish of 1 in 1,000 solution of mercuric chloride in water or 5 per cent. lysol kept on the bench for this purpose (in the case of tubes the plugs should be put in separately) and left for at least one hour. Then the glass-ware should be thoroughly washed in repeated changes of tap water to remove the last traces of antiseptic, a minute quantity of which will inhibit growth. Discarded cultures ought to be steamed in the Koch steriliser for two to three hours or autoclaved, in certain cases the media may be 'reclaimed' (p. 744). When a large number of tubes are being put out of use, they may be placed in an enamelled iron pail and this when full is sterilised in the Koch or autoclave. Cultures and other materials containing sporing organisms ought to be autoclaved. Pipettes and other glass-ware contaminated with spores may be immersed in concentrated sulphuric acid and afterwards thoroughly washed with water. If any film remains on the glass, e.g. in pipettes, it should be removed by soaking in bichromate nitric acid mixture (p. 775). In assembling glass-ware with metal caps after cleaning, e.g. universal containers, new 'liners' should be inserted. Infected tissues may be autoclaved or, if bulky, incinerated.

Cleaning of used slides. It should be noted that bacteria in films prepared in the usual way and stained with simple watery stains may still be alive; accordingly suitable precautions should be taken in handling such preparations. If the preparations have

are treated as if new (p. 775). It is advisable to discard all slides on which tubercle bacilli are present.

PRECAUTIONS TO BE TAKEN BY THE LABORATORY WORKER

An overall should be worn at work. Care must be taken not to contaminate the hands (the likelihood that the outside of tubes, etc., containing infective materials may have been soiled, should always be borne in mind). If infective tissues are handled

strong antiseptic treated similarly. work Nothing which has touched the laboratory bench or floor should be brought into contact with the mouth, thus it is dangerous to lick labels.

II

MICROSCOPIC METHODS

The Microscope. For ordinary bacteriological work a good microscope is essential. It ought to have a heavy stand, with coarse and fine adjustments, a double mirror (flat on one side, concave on the other), a good condenser with an iris diaphragm, and a triple nosepiece. The length of the microscope should be 160 mm (usually, in some cases 170 mm). It is preferable to have two eyepieces ($\times 5$ and $\times 10$). A binocular microscope is preferred by many, with this $\times 6$ or $\times 8$ eyepieces are suitable. Artificial illumination—some form of microscope lamp *e.g.* electric—is usually preferable to daylight. In the most recent forms the illuminant is incorporated. To adjust the condenser, place a microscope preparation on the stage and focus with the low-power objective, using the flat side of the mirror. Then, looking through the eyepiece, turn the condenser

length should be specified by the makers and includes the nosepiece—it is 160 mm (usually, in some cases 170 mm). It is preferable to have two eyepieces ($\times 5$ and $\times 10$). A binocular microscope is preferred by many, with this $\times 6$ or $\times 8$ eyepieces are suitable. Artificial illumination—some form of microscope lamp *e.g.* electric—is usually preferable to daylight. In the most recent forms the illuminant is incorporated. To adjust the condenser, place a microscope preparation on the stage and focus with the low-power objective, using the flat side of the mirror. Then, looking through the eyepiece, turn the condenser

then almost at the proper level for use with the immersion lens.

Use of the oil immersion lens. After the light is satisfactorily arranged, fix the slide on the stage with the right-hand clip and place a drop of immersion oil on the preparation. Lower the objective till the point touches the drop of oil. Then, rack the tube of the microscope slowly down till the objective dips into the oil. Now apply the eye to the

microscope is not fitted with a movable stage, it is convenient to use the left hand for moving the preparation whilst the right operates the fine adjustment. When the observer has finished for the time being with the immersion lens the oil ought to be wiped off with a piece of silk or lens paper. If the oil has dried on the lens it may be moistened with xylol.

MICROSCOPIC EXAMINATION OF BACTERIA

Micro-organisms may be examined (1) alive or dead in fluids, by wet preparations or hanging-drop preparations, (2) in film preparations, (3) in sections of tissues. In the two last cases advantage is always taken of the affinity of bacteria for certain stains.

Wet Preparations and Hanging-drop Preparations. In order to examine the appearance of organisms in aqueous fluids, *e.g.* to ascertain whether they are motile, a wet preparation is often sufficient. A loopful of the fluid is placed on a microscope slide and a No. 1 cover-glass¹ lowered on to it, so that a thin film forms between the two. For more extended study hanging-drop preparations are required (p. 758). When organisms are examined in fluids, it is necessary to adjust the condenser as usual but to use a small aperture of the condenser-diaphragm. It is best to focus the edge of the drop of fluid with a low-power objective, and—the preparation being arranged so that part of the edge crosses the centre of the field—to fix the slide in this position with a clip. A high-power lens is then turned into position, and lowered by the coarse adjustment to a short distance above its focal distance. It is now carefully screwed down by the fine adjustment, the eye being kept at the ocular meanwhile. The shadow of the edge will be first recognised, and then the bacteria must be carefully looked for. Often a dry lens is sufficient, but for some purposes the oil immersion is required. If the bacteria are small and motile, a beginner may have great difficulty in seeing them, and it is well to practise at first on

¹ A work on the microscope should be consulted.

² In bacteriological work it is essential that cover-glasses of No. 1 thickness ($1 \cdot 0$ to $1 \cdot 1$ mm thick) should be used, as those of greater thickness are not suitable for a $\frac{1}{2}$ in. lens.

some large non-motile form, such as *B. mycoides*. To find whether or not bacteria are motile, it is advisable to use either broth or agar cultures not more than twenty-four hours old, or preferably younger. Often motility is best observed in cultures grown at room temperature.

movement, the condition to be fulfilled is that the

are present in all motile forms except for observing the rate and mode of

Dark-ground illumination. The principle of this method is that the object to be examined is illuminated by rays of light brought to a focus on the object obliquely, no direct rays from the source of illumination reaching the eye of the observer. The object

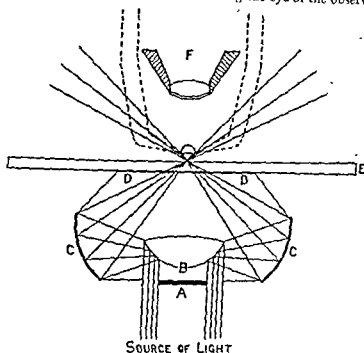


FIG. 222. Diagram showing course of rays in dark-ground illumination.

A Circular stop, B and C Reflecting surfaces, D Drop of oil between condenser and slide, E Microscope slide, J Lens

is thus seen brightly lighted on a dark background. A special form of condenser is required, which may be of the spherical or paraboloid type. In the former, the central rays of the beam of light are interrupted by a circular diaphragm while the lateral rays are reflected outwards from the lower surface of a spherical reflector and then inwards from another reflecting surface, and are brought to a focus in the position of the object to be examined (Fig. 222). A stop is placed within the oil immersion lens so as to cut down its N.A. to 1 or less—otherwise the dark-ground effect will not be obtained. The source of light is a 100 watt projection lamp, with a condensing lens which forms a beam of approximately parallel rays. The following are the steps in the procedure.

(1) The microscope with special condenser is placed in front of the source of light so that the beam of light occupies the surface of the substage mirror, the flat surface being used and the mirror being placed at an angle to direct the vertical rays of light upwards in the optical axis. (2) Centre the illumination. To do this use a low-power ocular; remove the condenser and swing out the objective. Then, holding a card or sheet of paper above the ocular, at right angles

le. Place a drop of
the examined, place

the latter on the stage and fix it with the clips. Then rack the condenser slowly up till the oil spreads in a uniform layer between the upper surface of the condenser and the

slide (Fig. 222). (4) Focus the preparation with a low-power objective. Rack the condenser a little till a bright spot of light is seen and then bring this by means of the centring screws of the condenser into the middle of the field. (5) Put a drop of oil on the cover-glass of the preparation. Place the immersion lens (fitted with the stop as described) in position and focus in the usual way. A little racking of the condenser or

on the surface of the condenser and proceed as in stages (3) to (5).)

It is a great advantage to use a separate microscope for dark-ground work and to have it mounted on a wooden stand along with the electric lamp and the condensing lens.

NOTE. Slides to be used for the dark-ground method ought not to exceed a thickness which is specified by the makers of the condenser (usually 1.2 mm.), if thicker, the light will be brought to a focus below the level of the film and the dark-ground effect will not be obtained.

Film Preparations. Dry method. This is the most extensively applicable method for the microscopic examination of bacteria. It depends on the fact that in the process of drying most bacteria undergo relatively little change in their forms. Fluids containing bacteria such as blood, pus, scrapings of organs, can be thus investigated, as also cultures in fluid and solid media. For all ordinary purposes films are made on slides, but sometimes cover-glasses are used. The first requisite is a perfectly clean slide or cover-glass. The test of this is that when the drop of fluid containing the bacteria is placed upon the glass, it can be uniformly spread with the platinum wire all over the surface without showing any tendency to retract into droplets. The best method is to place the cover-glasses or slides for some hours in a mixture of concentrated nitric acid 6 parts, potassium bichromate 6 parts, water 100 parts. After thorough washing in running water they may be kept in 60 per cent alcohol, for use, they are dried with a soft clean cloth. Slides may be rapidly cleaned by rubbing the surface with a moist cloth which has first been rubbed on a fine abrasive soap or powder (which, however, must not scratch the glass), then washing quickly under the tap and drying with a clean rag—this method must not be used with slides intended for dark-ground examination.

If a fluid is to be examined, a loopful may be placed on the glass and spread out over the surface with the wire. When a culture on a solid medium is to be examined, a loopful of water is placed on the slide, and a minute particle of growth rubbed up in it and spread out, so as to form a thin film. The usual mistake made by beginners is to take too much of the growth. It is sufficient just to touch the surface of the culture with the edge of the loop or the point of the straight wire, and when this is rubbed up in the drop of water and the film dried, there should be an opaque cloud just visible on the slide. In the case of *pus* or *sputum* a loopful should be spread out on the slide so as to make streaks of varying thickness, this may be done either by moving the looped wire from side to side or in a circular fashion. When a film has been spread it must next be dried at room temperature or by being moved backwards and forwards above a Bunsen flame, but not heated to a temperature higher than the hand can bear. The film must then be fixed on the glass, in the case of a slide, the underside is heated in

test-tube set in a rack

(a) in methyl alcohol (methanol) for two to three minutes, then washed and dried; (b) in formol-alcohol (Gulland)—formalin 1 part, absolute alcohol 9 parts—for five minutes, then washed and dried. In using the Romanowsky stains no previous fixation is necessary (*vide infra*).

Transmission of unfixed blood films from the tropics. In a cylindrical metal box a linen bag containing anhydrous CaCl_2 is placed, then a pad of cotton wool and lastly the slides. The lid of the box is fixed so as to be airtight, e.g. by luting with indiarubber grease mixture.

In the case of *urine*, the specimen is centrifuged and films are made from the deposit. It is an advantage to place a drop of distilled water on the dried film and heat gently to dissolve the deposit of salts; then wash gently in water and dry. In this way a much clearer picture is obtained when the preparation is stained.

Wet method. If it is desired to examine the histological structure of the cells of a discharge as well as to investigate the bacteria present, it is advisable to substitute wet-fixed films for the dried films, the preparation of which has been described. The nuclear structure, mitotic figures, etc., are by this method well preserved, whereas these are considerably distorted in dried films.

(a) Saturated solution of mercury perchloride in water. Fix for five minutes. Then rinse the films in water and thereafter wash in successive strengths of methylated spirit; treat with Gram's iodine to remove excess of mercuric salt, and again with spirit. After this treatment the films are stained and treated as if they were sections.

(b) Formol-alcohol (*vide supra*). Fix films for five minutes; then wash well in methylated spirit and water. They are then ready for staining. This is an excellent and very rapid method.

(c) Corrosive-alcohol—alcohol 1 part, saturated solution of mercury perchloride 2 parts. Fix for five minutes, wash with 50 per cent spirit, and treat as in (a). This fixative is very suitable for films of faeces containing entamoebæ.

Films prepared by the above methods are now ready to be stained by the methods described below.

Impression Preparations are used for studying whole colonies of bacteria and also for delicate organisms, such as those of the pleuropneumonia group, which do not withstand the ordinary methods of making films. A piece of solid medium 2 mm thick

cover-glass preparation stained and mounted (for details see Kieneneberger).

Sections of Tissues. The methods of fixing tissues will be described below, for the further stages in the preparation of sections, books on histological methods should be consulted. Paraffin sections give by far the best results; the procedure with these is as follows. Several drops of xylol are placed on the section and made to move backwards and forwards, the xylol is poured off and the process repeated. The excess of xylol is then removed by a clean cloth, care being taken not to allow the preparation to dry. A few drops of absolute alcohol are allowed to run over the preparation, so as to remove the xylol. This is followed in the same way by methylated spirit, and the slide is then placed in water. The section is then ready for staining. In the following account

hydration is usually effected by alcohol. The preparation, which has been brought into water, is treated with a few drops of alcohol and this is repeated several times, then cleared in xylol, and mounted in a suitable mounting medium—B P S* is preferable to Canada

* Absolute industrial methylated spirit (74 O P) serves as a substitute for absolute alcohol for many staining and histological purposes. Industrial methylated spirit of the type known as 'toilet spirit, acetone free (66 O P)', which does not contain enough mineral naphtha to cause it to become milky on addition of water, serves instead of rectified spirit. These industrial spirits are relatively cheap; under permit from the Customs authorities they can be purchased duty-free.

* Kirkpatrick and Lendrum's B P S mounting medium is prepared as follows. 5 c.c. of dibutylphthalate (B D H) are mixed with 35 c.c. of xylol, in this are dissolved 10 grms. of Distrene 80 (British Resin Products Ltd., Greig & Burgh, Epsom, Surrey). The synthetic resin preserves the colours of bacteriological preparations very much better than balsam. When using this mountant it is essential to remove from the section all traces of paraffin with fresh xylol.

balsam. After being treated with xylol the preparation ought to be perfectly clear, if any opacity remains, further dehydration with alcohol is necessary. For certain purposes, e.g. when the bacteria are readily decolorised by alcohol, aniline-xylol (aniline 2 parts, xylol 1 part) is used as the dehydrating agent. The preparation is quickly dried with blotting-paper, then treated with aniline-xylol which clears at the same time. The aniline-xylol is then replaced by xylol, this is conveniently done by running the xylol over the preparation in one direction, when the aniline-xylol will be seen to be displaced before it. The preparation is then mounted.

Sections of colonies. A piece of solid medium bearing a colony is treated as if it were tissue (see Klieneberger).

THE STAINING OF BACTERIA ETC.

In general the protoplasm of bacteria, like the nuclear chromatin of animal and plant cells, has an affinity for basic aniline dyes. Aniline dyes, which have the constitution of salts, are divided into two groups according as the staining action depends on the basic or the acid portion of the molecule. Thus the acetate of rosaniline derives its staining action from the rosaniline, it is therefore called a basic dye. On the other hand, ammonium picrate owes its action to the picric acid part of the molecule, it is therefore termed an acid dye. Certain acid aniline dyes, e.g. eosin or rose bengal, also stain bacteria.

The following are the most commonly used basic stains (the figures in brackets after each indicate the number in the Colour Index (C.I.) of the Society of Dyers and Colourists¹ and the approximate percentages soluble in 95 per cent alcohol and in water respectively.)

Violet Stains. Crystal violet chloride (C.I. No. 681 14, 17) and the closely related methyl violet (C.I. No. 680 15, 3) and gentian violet.

Blue Stains. Methylene blue, chloride (C.I. No. 922 15, 36) (synonym phenylene blue), Thionin (C.I. No. 920 025, 025) (also known as Lauth's violet), Toluidine blue (C.I. No. 925 057, 38).

Red Stains. Basic fuchsin, chloride (C.I. No. 677 8, 04) (synonyms basic rubin, magenta, rosanilin), Safranin (C.I. No. 841 34, 55) (synonyms fuchsia giroflé).

Brown Stains. Bismarck-brown (C.I. No. 331 1, 14) (synonyms vesuvium, phenylene brown), Chrysoidin (C.I. No. 20 22, 086).

Of the stains specified the violets and reds are the most intense in action, especially the former, it is thus easy in using them to over-stain a specimen. It is difficult to over-stain with methylene blue. Thionin also gives good differentiation and does not readily over-stain.

It is most convenient to keep saturated alcoholic solutions of the stains made up

in a short time in such fluids

The staining of films. Films are made on slides from cultures as described above and a few drops of the stain are placed on the surface. When the film has been stained for the requisite time it is well washed with soft tap water or distilled water, surplus water is drawn off with a piece of filter-paper or wiped off round about the preparation and it is carefully dried high over a flame, a drop of immersion oil is placed on the film and it is examined. If the preparation is to be preserved the immersion oil should be removed by xylol which is then drained off. Such a preparation if kept free from dust, e.g. by wrapping in a piece of glass paper, or it may be mounted

fluids if the histological elements also claim attention it is best to stain first the cellular

¹ Bradford 1924, Supplement, 1928

carbol-fuchsin will be found to be the best; the Gram method (*vide infra*) is also essential, and subsequently any special stains which may appear advisable.

Staining of sections may be carried out by the procedures described below. When staining is to be prolonged, sections should be fixed to the slide, otherwise there is the danger of their becoming detached. Masson's method of fixation is recommended; the section is flattened in the usual way on a clean slide by floating it on several drops of a warm 1 in 1,000 solution of gelatin in water, which is preserved with a crystal of thymol; then the excess is drained off and the slide, standing on its end, is exposed to the vapour of formalin in a closed jar at 40° to 50° C overnight. Sections from tissues fixed with mercuric chloride contain a dark deposit which is removed before staining by treatment with iodine (1 part of iodine and 2 parts of potassium iodide in 150 parts of water), the process being controlled by examination with the low power of the microscope; the iodine is then removed with 5 per cent. aqueous solution of sodium thiosulphate followed by rinsing in water. Carbol-thionin is a very useful stain for demonstrating bacteria. The modification of the method of staining by the use of a mordant is also possible. The advantage of this is that the tissue elements may be stained to such an extent as to obscure the bacteria. Hence many methods have been devised in which the general principle consists in (a) the use of a penetrating agent or mordant which increases the staining power and tends to fix the stain in the bacteria, e.g. phenol, aniline, metallic salts, weak solutions of alkalis, heat, prolonged action of the stain; or (b) subsequent treatment by substances which decolorise the overstained tissues to a greater or less extent, while they leave the bacteria coloured, e.g. dilute acids—hydrochloric, acetic, etc; alcohol either as methylated spirit or absolute alcohol; or a combination of spirit and acid—methylated spirit with 1 per cent. of hydrochloric acid added; also aniline and various other substances which have been sufficiently decolorised.

with 2.5 per cent ammonium molybdate in water. Different organisms stain differently, and thus duration of staining and decolorisation should be varied accordingly.

Bacteriological Stains

The following stains are commonly used

Löffler's methylene blue

Saturated solution of methylene blue in alcohol 30 cc

Solution of potassium hydroxide in distilled water (about 1 : 10,000) 100 "

The rate and intensity of staining depend largely on the amount of alkali used. Films may be stained by five minutes' exposure or longer in the cold. They usually do not require decolorisation, as the tissue elements are not over-stained. Sections may be stained in this mixture for from a quarter of an hour to several hours. They do not readily over-stain. The tissue containing the bacteria is then decolorised if necessary with $\frac{1}{4}$ to 1 per cent acetic acid, till it is a pale blue-green. The section is washed in water, rapidly dehydrated with alcohol or aniline-xylol, cleared in xylol, and mounted.

Polychrome methylene blue This is a methylene blue solution which has been 'ripened' by oxidation so that new violet compounds are formed which stain certain structures, e.g. the granules in *B. diphtheriae*. Such a solution may be conveniently made from Löffler's methylene blue by shaking a half-filled bottle from time to time over a long period, several months are necessary to complete the process.

Unna's polychrome methylene blue solution has the following composition.

methylene blue	1 gm.
potassium carbonate	1 "
distilled water	100 cc

It is ripened by the method just described and for use is diluted with 5 to 10 volumes of water.

Carbol-thionin Heat 1 gm thionin in 500 cc water in the Koch at 100° C for thirty minutes; cool and add 500 cc of 1 per cent solution of phenol in water, filter. The solution keeps indefinitely.

¹ Polychrome methylene blue is obtainable commercially.

Stain films or sections for three to five minutes. If more prolonged staining is required, the stain must be washed off with water and a fresh portion filtered on, since on prolonged contact insoluble crystals form on films or sections. This is especially marked if the latter contain formalin deposit, the sections should then be treated with absolute alcohol saturated with picric acid before staining. Differentiation, if necessary, may be effected by brief treatment with methylated spirit followed by absolute alcohol and xylol.

Rose bengal 1 per cent in 5 per cent watery solution of phenol is useful for staining selectively the bacterial bodies where much capsular material is present.

Ziehl-Neelsen's carbol-fuchsin. This has the composition

basic fuchsin (powdered)	10 grms
alcohol	100 c c
phenol (carbolic acid crystals)	50 grms
water (distilled)	1,000 c c

Place the phenol in a flask along with the fuchsin. Mix thoroughly and heat on a boiling water-bath for five minutes shaking occasionally, complete solution occurs. Add the alcohol and mix well. Finally add the water. This is a very powerful stain and in the undiluted condition is chiefly used for staining acid fast bacilli (p. 781).

Dilute carbol-fuchsin is *Ziehl-Neelsen's* carbol fuchsin diluted with twenty times its volume of water or more. Stain for ten to fifteen seconds. The dilute carbol-fuchsin should be discarded when it ceases to be quite transparent.

Crystal violet as a 0.5 per cent solution in water is useful for organisms which tend to stain weakly with other dyes, e.g. *V. cholerae*.

Martinotti's stain for fungus in hairs and skin scales. Dissolve lithium carbonate 0.5 gram and toluidine blue 1.0 gram in 75 c c distilled water, then add 20 c c glycerol and 5 c c methylated spirit.

Fix the scales or hairs on the slide with 50 per cent glacial acetic acid and dry at 37°C. Wash in water and treat with ether to remove fat then

- (1) stain for five minutes,
- (2) wash in water,
- (3) differentiate with 0.5 per cent acetic acid,
- (4) dehydrate with water-free acetone (two to three minutes),
- (5) clear with xylol and mount.

Staining both of organisms and tissue elements in sections. *Stafford's* method (modified) is simple and satisfactory. (a) treat the section for three minutes with a 1 in 5 dilution in water of the following stain—potassium bichromate 1 gram, eosin 'water-soluble yellow', 1 gram water 100 c c, (b) wash in water (half a minute) and counter stain with 1 per cent aqueous methylene blue for one minute, (c) wash in water, decolorise in methylated spirit, dehydrate in absolute alcohol, clear in xylol and mount.

Erythrosin orange-toluidine blue (*Masson*). Sections of tissue fixed in *Zenker's* solution are mordanted with *Gram's* iodine for thirty minutes, bleached with 5 per cent aqueous sodium thiosulphate, rinsed in running water and stained for ten to fifteen

absolute alcohol, cleared with xylol and mounted. This stains organisms (blue) and tissue elements

Gram's Method and its Modifications

bacteria. The stain used is a triphenylmethane dye, crystal violet (or an analogous compound, methyl violet or gentian violet). The essential step in the method is the treatment of the preparation with a solution of iodine after staining. The iodine forms with the stain in the bacteria a compound which resists decolorisation with such agents as alcohol, aniline-xylol, acetone, etc., whilst the tissues are decolorised and can then be coloured by a contrast stain.

but sometimes there is great variability in the avidity with which organisms stained by Gram's method retain the dye when treated with the decolorising agent, and it may be difficult to say whether an organism is Gram-positive or not. Such variations may be due to the state of the organisms, the commonest variation being for a Gram-positive organism to become in older cultures Gram-negative or for a degenerate organism, *e.g.* in pus, to become decolorised. But the result frequently depends on the procedure followed in staining; prolonged action of the decolorising agent, or the presence of a minute trace of water, *e.g.* in aniline-xylol, intensifies its action. It must also be mentioned that fibrin and some tissue elements may retain the stain as firmly as any bacteria.

fresh iodine solution be used. (In some of the modifications iodine is dispensed with) The following have been selected as suitable:

Weigert's Modification

The following are the solutions necessary:

(1) Crystal violet (or methyl violet 6 B) 0.5 per cent solution in water (this is preferable to the original dye solution—carbol-gentian-violet—consisting of a mixture of saturated alcoholic solution of gentian violet 1 part *plus* 5 per cent aqueous solution of phenol 10 parts)

(2) Gram's iodine solution.

iodine	1 grm
potassium iodide	2 grms.
distilled water	300 c c.

(3) Aniline-xylol

aniline	2 parts
xylol	1 part.

(4) Dilute carbol-fuchsin (p. 779)

FILMS after being fixed are treated as follows.

(1) Stain with violet solution for one to two minutes

(2) Pour off stain and, without washing, add Gram's iodine solution, allow to act for one to two minutes

(3) Pour off iodine and dry with blotting-paper

(4) Decolorise with aniline-xylol till the preparation is of a pale violet colour. In the case of films of pus, etc., or sections, decolorising should be controlled under the low power of the microscope till the nuclei have a faint violet colour (the nuclei sometimes retain the violet stain very tenaciously, decolorising may then be aided by breathing slightly on the film while covered with aniline-xylol). Note that thick parts of a smear of pus etc., will not decolorise and are in any case unsuitable for examination

(5) Wash off aniline-xylol thoroughly by means of xylol and then allow to dry.

(6) Counter-stain with dilute carbol-fuchsin for about ten to twenty seconds. Wash in water and dry. Avoid over-staining with the counter-stain, since dark red organisms (Gram-negative) may be difficult to distinguish from those which retain Gram's stain. For demonstrating gonococci neutral red 0.1 per cent and acetic acid 0.2 per cent in water is a good counter-stain

Alternative procedure stages (1) and (2) as above

(3) Wash quickly with water

(4) Decolorise by flooding the film with 'industrial spirit', several changes being used until further stain ceases to be removed, or the film may be rapidly treated with acetone

(5) Wash quickly with water. Then control the result of decolorising with the low power of the microscope as in (4) above, if necessary, repeat stages (4) and (5)

(6) Counter-stain, etc., as in (6) above

Control spot In order to avoid errors from over-decolorising when examining pus for Gram-negative organisms, it is advisable to make a small film of a known Gram-positive organism (from a twenty-four hours' pure culture, *e.g.* of staphylococcus) at one side of the pus smear, this 'control spot' is then treated in the same way as the rest of the film. For the diagnosis of Gram-negative organisms, *e.g.* gonococcus or meningococcus in pus, it is essential that the 'control spot' should retain the Gram's stain vigorously, while the nuclei of the pus cells are at the same time stained pink

SECTIONS should be first stained by carmalum-cochineal¹ for ten minutes. The steps of procedure are then the same as the above, aniline-xylol being used for decolorising, but in stage (5), after being treated with xylol, the preparation is mounted.

Hucker's Modification for Fixed Films

Mix one part of a 10 per cent solution of crystal violet in 95 per cent ethyl alcohol with 4 parts of a 1 per cent aqueous solution of ammonium oxalate. Sometimes after staining with this mixture Gram negative organisms e.g. the gonococcus are difficult to decolorise. In this event the crystal violet solution may be diluted up to ten times before mixing in the same proportions. Stain the smear for one minute with the crystal violet solution, wash in water, treat with Gram's iodine solution for one minute, wash in water and blot dry. Decolorise with 95 per cent ethyl alcohol for thirty seconds, agitating gently. Blot and counterstain with Safranin O (10 c.c. of a 2.5 per cent alcoholic solution of the dye in 100 c.c. of water) for ten seconds then wash and dry.

Kopeloff and Beerman's Modification

FILMS are allowed to dry in air, fixed with the least amount of heat necessary, and treated as follows:

(1) Flood with a freshly prepared mixture of 1 per cent aqueous crystal violet solution, 15 parts, with 5 per cent solution of sodium bicarbonate 4 parts and allow to stand for five minutes.

(2) Flush off the excess of stain with iodine solution (2 grms iodine, 10 c.c. N/1 NaOH, made up to 100 c.c. with distilled water) then cover with fresh iodine solution and let stand two minutes or longer.

(3) Blot off all free water, but do not wash or dry.

(4) Decolorise with acetone until the decoloriser flows from the slide practically uncoloured (this usually takes less than ten seconds).

(5) Dry in air.

(6) Counterstain for ten to thirty seconds with dilute carbol fuchsin—1:20.

(7) Wash in water, blot and dry.

In the case of films of pathological material this method should be used for demonstration purposes rather than for determining how an unknown organism reacts to Gram's method, as occasionally coliform bacilli in urinary sediment have appeared to be Gram-positive. This fallacy has not been met with in staining cultures.

SECTIONS (according to Dunlop) are treated as above at stages (1) to (4), then proceed as follows:

(a) Wash in water.

(b) Counterstain with Ziehl-Neelsen carbol-fuchsin diluted 1:250 with water, for two minutes or longer.

(c) Wash in water.

(d) Dehydrate quickly with methylated spirit followed by absolute alcohol. Since alcohol tends to remove the fuchsin rapidly an alternative procedure is, after wiping off excess of water round the section, to dehydrate with a mixture of 'cellosolve' 1 vol. and xylol 2 vols. (the section after sufficient treatment should be almost clear).

(e) Clear in xylol and mount.

Kirkpatrick's Method for Sections

This method gives very satisfactory results with sections containing Gram positive organisms, especially where there is difficulty in decolorising the tissues by other methods, fibrin is not positively stained.

1. Stain with carmalum-cochineal for ten minutes, wash in water for a few minutes.

2. Flood with 0.5 per cent crystal violet for two minutes, wash in water.

11

Stain for Tubercle and other Acid-fast Bacilli

Such organisms cannot be well stained with a simple watery solution of a basic aniline dye although with the Gram method a partial staining is effected. They require

water 200 c.c.,
in the Koch for

a powerful stain containing a mordant, and must be exposed to the stain for a long time, or its action may be aided by a short application of heat. A mixture of crystal violet or basic fuchsin with aniline or phenol is suitable. When once stained these organisms resist decolorising even with strong acids; they are therefore called 'acid-fast'. The following method is most commonly used.

1. Filter and wash.

c
f
n
s

Wash not stain, loose sections with cold; in hot stain the latter shrink.) Wash in running cold water for about a minute.

2. Decolorise with 90% (nitric acid or hyd

3. Wash in water.

Wash in spirit for half a minute, and replace in water.

4. Counterstain with an aqueous 0.5 per cent solution of methylene blue for half a minute.

5. Wash well with water. In the case of films, dry. In the case of sections, dehydrate, clear, and mount.

In specimens stained by the above method acid-fast bacilli are bright red. Other bacteria which may be present, and cells or tissues, are coloured with the counter-stain (blue).

Tubercle bacilli, which are intensely acid-fast, retain the stain even after treatment with the acid for many hours; but other acid-fast bacilli may become decolorised in a few minutes. Some acid-fast the brief treatment with alcohol that coliform organisms which resist the decolorising processes with the staining of acid-fast bacilli in sections, etc. To remedy this, use a staining solution which has been brought to pH 8.0 immediately before use by adding sodium bicarbonate solution (Fielding), excess of alkali must be avoided as it causes precipitation of the stain.

Leprosy bacilli are stained in the same way, but are often rather more easily decolorised than tubercle bacilli, and it is better to use only 0.5 per cent. sulphuric acid in decolorising.

A staining solution containing crystal violet, instead of basic fuchsin, may be used by those who have difficulty in discriminating between red and blue tints. In this case chrysoidin (1:300 in water) is used as the counter-stain.

Stains for the Diphtheria Bacillus.

Diphtheria bacilli have a strong affinity for methylene blue and a 0.5 per cent aqueous solution applied to films for several minutes demonstrates the intensely stained granules (volutin granules) in the body of pale blue bacilli. The following methods bring out the structural characters of this organism. In the case of cultures, films should be made from eighteen to twenty-four hours' growths on Löffler's serum medium.

Neisser's Stain (second or modified method)

1. Stain films for a few seconds in a mixture of solutions A and B, two parts of the former to one of the latter.

A	methylene blue	1 grm
	absolute alcohol	50 c c
	glacial acetic acid	50 "
	distilled water	1,000 "
B	crystal violet	1 grm
	absolute alcohol	10 c c
	distilled water	300 "

2. Wash for a few seconds in soft or distilled water (this stage may be omitted).

3 Stain in chrysoidin solution (1 : 300) for a few seconds (the chrysoidin should be dissolved in warm water and the solution then filtered)

4. Wash quickly in water, blot, and dry

The substance of the bacilli is brownish yellow, the granules are almost black

Instead of chrysoidin the following solution of erythrosin may be used Saturated, alcoholic solution of erythrosin, 20 parts, saturated watery solution of picric acid, 90 parts, add to the mixture precipitated calcium carbonate to excess, allow to stand for a time, shaking at intervals filter

Pugh's Stain

The staining solution consists of

toluidine blue	0.1 gm
absolute alcohol	2 c c
5 per cent solution of glacial acetic acid in distilled water	100

Stain for two to three minutes Wash with water and dry The substance of the diphtheria bacilli is light blue and the granules are of a reddish purple tint

Albert's Stain (modified by Laybourn)

Dissolve 0.15 gm toluidine blue and 0.2 gm malachite green in 2 c c 95 per cent alcohol, then add 100 c c distilled water and 1 c c glacial acetic acid allow to stand for one day then filter

Iodine 0.66 per cent, and potassium iodide 1 per cent in distilled water

Films after fixation by heat are treated with the stain for three to five minutes washed in water, then treated with the iodine solution for one minute, washed in water and blotted dry

The granules of diphtheria bacilli are bluish-black the protoplasm green, and other organisms chiefly light green

Staining of Spores

If bacilli containing spores are stained with a watery solution of a basic aniline dye the spores remain unstained The spores either take up the stain less readily than the protoplasm of the bacilli, or they have a resisting envelope which prevents the stain from penetrating to the protoplasm When once stained they are less readily decolorised than the bodies of the bacilli In fixing the films little heat should be used, as overheating interferes with the subsequent staining The following is a simple method for staining spores

- 1 Stain films with carbol-fuchsin (p 779), heating as for tubercle bacilli
- 2 Wash in water and treat with 30 per cent solution of ferric chloride for one to two minutes
- 3 Without washing treat with 5 per cent sodium sulphite solution for fifteen to thirty seconds (according to Armstrong)
- 4 Wash in water
- 5 Stain with 1 per cent aqueous methylene blue for half to one minute
- 6 Wash in water, and dry

The spores are stained red, the protoplasm of the bacilli blue

The spores of some organisms lose the stain more readily than those of others

Negative Staining

Nigrosin method (Dorner) Nigrosin (water soluble) 10 grms are dissolved in 100 c c distilled water by boiling for thirty minutes, then 0.5 c c formalin is added as a preservative The mixture is filtered twice through the same double filter paper and distributed in 5 c c amounts in containers On a slide mix one loopful of the stain with one loopful of bacterial suspension, spread, and allow to dry Then examine in immersion oil (Acid precipitates the nigrosin, therefore an acid culture must first be neutralised)

Staining of Capsules

Anthony's Method

Smears are dried in air and, without fixation, stained with 1 per cent solution of crystal violet in water, then washed with 20 per cent aqueous copper sulphate and blotted dry

Hiss's Method

The organisms should be suspended in serum or a medium containing serum or ascitic fluid. Films are allowed to dry, fixed, and then stained with a mixture of 1 or 2 volumes of saturated alcoholic solution of gentian violet or basic fuchsin plus 10 volumes of water, being heated gently over a flame for a few seconds till steam rises. The dye is washed off with 20 per cent. copper sulphate solution and the film blotted dry.

Richard Muir's Method (modified)

1 The film containing the bacteria must be very thin. It is dried and stained in filtered carbol-fuchsin for half a minute, the preparation being gently heated.

2 Wash slightly with spirit and then well in water.

3 Place in the following mordant for a few seconds:

saturated solution of corrosive sublimate	2 parts
tannic acid solution—20 per cent	2 "
saturated solution of potash alum	3 "

4 Wash well in water.

5 Treat with methylated spirit for about a minute. The preparation has a pale reddish appearance.

6 Wash well in water.

7 Counter-stain with ordinary watery solution of methylene blue for half a minute.

8 Dehydrate in alcohol, clear in xylol, and mount.

The bacteria are a deep crimson, and the capsules of a blue tint.

For a method of staining capsules of bacteria in sections, reference may be made to Rd Muir, *Journ Path and Bact*, 1916, 20, 257.

Kirkpatrick's Method

Fixing solution—

formalin	10 c c
chloroform	30 "
absolute alcohol	60 "

Manson's borax methylene blue—

methylene blue	1 gm
borax	2 grms.
water	100 c c

Dissolve the borax in boiling water over a flame, add methylene blue, cool, and filter. For use take

borax methylene blue	1 part
distilled water	5 parts.

(1) Make a film on a slide, then allow to dry in air; the film must not be heated.

(2) Pour on fixing solution and allow to remain for one to three minutes.

(3) Wash off with spirit; drain off excess and stain with dilute borax methylene blue for one to three minutes.

(4) Wash quickly in water, blot, and dry, or dehydrate in acetone, clear in xylol, and mount.

Relief or Negative Staining

Staining solution (Howie and Kirkpatrick's method):

- | | |
|--|---------|
| (1) 10 per cent water-soluble eosin ('yellowish' or 'black') | 4 parts |
| or erythrosin in distilled water | |
| (2) serum (human, rabbit, sheep or ox, heated at 56°C) | 1 part |
| (3) crystal of thymol | |

The mixture is allowed to stand at room temperature for several days and is then centrifuged and the supernatant fluid stored at room temperature; it keeps for many months.

On a clean slide, with a platinum loop of about 1 mm diameter, one drop of exudate (or fluid culture or a suspension in broth from an agar slope culture) is mixed with one drop c
a min.
After t

examined in immersion oil. The appearances do not alter when the films are kept for many months. (In the case of cocci the treatment with Ziehl-Neelsen's solution can usually be omitted.)

Capsules of cocci are demonstrated by mixing on a microscope slide one loopful of exudate containing the organisms or culture with one loopful each of 1 per cent aqueous solutions of rose bengal and nigrosin, mixing thoroughly and spreading as a film. The bodies of the cocci are pink, capsules white, and the background slate coloured.

Staining of Flagella

The staining of the flagella of bacteria requires considerable practice to ensure good results. Many methods have been introduced, of which the two following are very satisfactory.

Kirkpatrick's Method

Solutions required

Fixing Solution

absolute alcohol	60 c.c.
chloroform	30
formalin	10

Mordant

ferric chloride, 5 per cent solution	1 part
tannic acid, 20 per cent solution (dissolved by heat and allowed to cool)	3 parts

Before use, dilute the mordant with an equal volume of water.

Silver Solution

Place 10 grms. of silver sulphate (B.D.H.) in a clean chemist's bottle and add 200 c.c. distilled water. Incubate at 37° C. for twenty-four hours, shaking occasionally. This stock solution improves by leaving it exposed to daylight and keeps indefinitely. Transfer a clean 100 c.c. Erlenmeyer flask with distilled water. Then add 10 c.c. of filtered silver stock solution in the flask and add quickly 0.6 c.c. ethylmagnesium iodide (W.S. B.D.H.). A precipitate forms which is immediately redissolved. Then add a pinch of iron filings (a piece of unused quill tubing) add filtered silver stock solution until a permanent slightly opalescent solution results. Finally, add 10 c.c. of distilled water.

Procedure

Bacterial suspension. In the case of the coli type of flagella, cultures which have been grown at 37° C. for forty-eight hours are best. For other species, twenty-four hours' cultures may be preferable. Heat a clean slide over the flame of the Bunsen flame and allow to cool. Place about 1 c.c. steril distilled water on the tube. With a platinum wire remove a loopful of the agar when taking care not to break the surface of the medium. Suspend the loopful of the culture in the distilled water and gently rotate the loop until an even suspension is obtained. Add distilled water to make 1 c.c.

5. films should be cleaned in the bi-

- (2) Place the film in fixing solution in a jar for one to three minutes.
- (3) Rinse in spirit, then wash thoroughly in water.
- (4) Treat with mordant for three to five minutes in a staining jar.

er on silver staining solution
dipped in methylated spirit

Continue heating, keeping the flame moving, until the control spot appears of a dark brown colour and a metallic scum forms on the edges of the fluid—about fifteen seconds. Cease flaming and allow the heated silver solution to act for fifteen to thirty seconds longer.

- (7) Wash off the staining solution in running water (the staining solution must not be poured off or deposit will form on the film).
- (8) Dry the film and mount.

A well-stained film shows the organisms uniformly black, the flagella being clearly defined and of a light brown-black or grey colour.

... staining while staining, or insufficient ethylamine in the staining solution. A silver staining solution which contains excess of ethylamine may be corrected by the addition of a few drops of the stock silver solution, one which contains too little ethylamine had better be rejected.

Pitfield's Method (modified by Richard Muir)

Prepare the following solutions :

Mordant :

tannic acid, 10 per cent, watery solution, filtered	10 c.c.
corrosive sublimate, saturated watery solution	5 "
alum, saturated watery solution	5 "
carbol-fuchsin (<i>vide</i> p. 779)	5 "

Mix thoroughly. A precipitate forms, which must be allowed to deposit, either by centrifuging or on standing. Remove the clear fluid with a pipette, and transfer to a clean bottle. The mordant keeps well for one or two weeks.

Stain :

alum, saturated watery solution	10 c.c.
gentian violet, saturated alcoholic solution	2 "

The stain should not be more than two or three days old when used. It may be substituted in the mordant in place of the carbol-fuchsin.

A bacterial suspension having been prepared as above described, a film is made by spreading a drop on a clean cover-glass and is allowed to dry in the air; it is then passed

twice or three times
of the
to rise,
water if

dry pour on some of the stain. Heat as before, allowing to steam for about a minute, wash well in water, dry, and mount.

Staining of Spirochætes in Films

Fontana's Method

Three solutions are required :

- Fixative*. Acetic acid 1 c.c., formalin 2 c.c., and water 100 c.c.
- Mordant*. 5 per cent. tannic acid in a 1 per cent. watery solution of phenol.
- Stain*. 0.25 per cent. solution of silver nitrate in distilled water. For use a small quantity of this is put in a test-tube, and a minute amount of ammonia solution is added till there is distinct turbidity. (If too much ammonia is added the fluid becomes clear again.)

solution (c) is dropped on, heated till steam rises, and allowed to remain for another half minute. The preparation is finally washed in water and dried.

The spirochætes are of a dark brown or black colour, and are easily found.

Becker's Method (modified)

The *Fixative* and *Mordant* used are the same as in Fontana's method (*vide supra*).

Staining Solution

basic fuchsin (saturated alcoholic solution)	45 c.c.
Shunk's mordant B (absolute alcohol or 95 per cent)	18 "
ethyl alcohol	
aniline	
distilled water	100 "

Mix the Shunk's mordant with the alcoholic fuchsin solution, then add the distilled water. The glass-ware should be thoroughly dry, or rinsed out with alcohol before use. The stain and reagents are filtered into jars for use.

Procedure

- (1) Make the film on a slide and allow to dry
- (2) Place in fixative for one and a half to three minutes
- (3) Wash in water (half a minute) and treat with mordant for three to five minutes.
- (4) Wash in water (half a minute) and place in staining solution for three to five minutes
- (5) Wash in water, dry, and mount.

Relief Staining

India-ink An emulsion of india-ink of fine quality is sterilised by steaming and allowed to settle for a few days, a drop of the deposit diluted with an equal quantity of distilled water, is well rubbed up, mixed with a drop of the material to be examined (exudate from chancre or condyloma, scraping from congenitally affected organ, etc.), and spread on a slide. The film is dried and examined with an immersion lens without the interposition of a cover. Spirochaetes, if present, stand out unstained, surrounded by the dark india-ink, and often positive results are rapidly obtained by means of it. The organisms are not so readily recognised by this method as by dark-ground illumination, and negative observations are thus less valuable.

Collargol, which should be shaken before use, 1 part, diluted with distilled water, 19 parts, is employed in a similar way to india-ink.

*Giemsa's Stain (p 789)***Staining of Spirochaetes in Tissues**

The following impregnation methods gave excellent results

Levaditi's Original Method

- (1) The tissue, which ought to be in thin slices, about 1 mm in thickness, is best fixed in 10 per cent formalin solution for twenty-four hours.
- (2) wash for an hour in water, and then bring into 96 per cent alcohol for twenty-four hours,
- (3) place in 1.5 per cent solution of silver nitrate in a dark bottle, and keep in an incubator at 37° C for three days,
- (4) wash in water for about twenty minutes, and thereafter place in the following mixture

pyrogalllic acid	4 grms
formalin	5 c c
distilled water up to	100 ..

Jahnel's Method for Nervous Tissue, etc

- (1) Wash in water for one to three days thin pieces (2 to 4 mm) of tissue which have been fixed in 10 per cent formalin for at least fourteen days.
- (2) place in pure pyridine for one to three days.
- (3) wash in many changes of water until the smell of pyridine is practically gone—two to three days.
- (4) place the pieces in 5 to 10 per cent formalin for a few days.
- (5) wash thoroughly in water.
- (6) place for half to one hour in fresh 1 per cent solution of uranium nitrate in distilled water at 37° C (the uranium nitrate prevents subsequent staining of other elements in nervous tissue), penetration is assisted by letting the tissue rest on a layer of lead-free glass wool.
- (7) wash for one day in distilled water.
- (8) place in 96 per cent alcohol for three to eight days.
- (9) wash in distilled water till the tissue sinks.
- (10) place in 1.5 per cent solution of silver nitrate crystals and leave five to eight days at 37° C. The container should be dark.

(11) decant off the silver nitrate solution, wash the tissue slightly in water in the dark, then transfer it for twenty-four to forty-eight hours at room temperature in the dark to a mixture made up as follows.

4 per cent. watery pyrogalllic acid 90 c.c. } reject 15 c.c. of this solution and re-
acetone " " 10 " } place by pyridine :

(12) wash in distilled water, dehydrate in neutral alcohol, clear and embed in paraffin

It should be noted that spirochaetes stained with silver rapidly become colourless on contact with certain immersion oils.

Nyka's Methods for Rickettsiae in Sections.

These methods are applicable to paraffin sections of tissues such as infected mouse lung

I For tissue (small pieces) fixed in Muller's fluid

- (1) stain for 5 to 10 minutes in a 5 per cent solution of basic fuchsin in 90 per cent. alcohol ;
- (2) rinse rapidly in tap water and treat with 90 per cent alcohol for 1 to 2 minutes ;
- (3) stain in 1 in 10,000 aqueous methyl violet for 1 to 3 minutes ;
- (4) rinse in tap water ; differentiate in 1 in 1,500 acetic acid ;
- (5) dehydrate in absolute alcohol or acetone, clear in xylol and mount

II For tissue fixed in 10 per cent. neutral formol proceed as follows :

- (1) stain for 30 to 60 minutes with 1 in 10,000 aqueous methyl violet ;
- (2) differentiate with water containing 2 drops of glacial acetic acid in 100 c.c. until the cytoplasm of cells is seen under the microscope to be colourless ;
- (3) counterstain for a few seconds with 1 in 10,000 aqueous metanil yellow ;
- (4) dehydrate with acetone, clear in xylol, and mount.

The Romanowsky Stain

This stain and its modifications are extensively used for demonstrating protozoa (*malaria parasites, trypanosomes etc.*), spirochaetes, Rickettsia bodies and viruses, and the blood cells, as which is formed when eosin are brought to alcohol. The stain

changes, such as occur in methylene blue solutions with age, are brought about by treatment with alkalis, especially

derivatives in combination proportions in which

of the staining fluid, account for the different effects produced on the contents of a cell. The underlying chemical reactions are complicated and as yet not fully understood. Thus it is not certainly known to what particular new body the reddish hue produced in chromatin is due, but the active constituent may be methylene violet or methylene azure or thionin, all of which result from the action of alkali on methylene blue. The following are the chief formulæ in use

as a solution already made up in alcohol. The mixture is shaken

ready for use. The stain will keep for

Films of blood, etc., are allowed to dry before being stained

- (1) Support the slide or cover-glass in a horizontal position raised off the bench
- (2) Drop on the stain from a pipette until the whole surface is uniformly covered

is usually specified. The important point is that the presence of 100 NaOH is fairly satisfactory provided that the pH is adjusted

(5 to 10 drops for a cover-glass, 20 to 30 drops for a slide) Allow to act for one and a half minutes

(3) With the same pipette add 1 or 2 drops of distilled water (or soft tap water) for each drop of stain used under (2) The fluids will mix thoroughly without any rocking of one

, or
the

(5) Drain off excess of water and allow the stained film to dry in air (do not heat)

(6) Mount

The use of a neutral buffer solution instead of water for washing is stated to give improved results in this and other Romanowsky methods. A solution sufficiently accurate for this purpose is made by dissolving KH_2PO_4 3.53 grms and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 14.58 grms in one litre of distilled water

The results are best with freshly prepared films or with such as are only a few days old. If older than this there is an increasing tendency for the red cells to stain of a dusky bluish colour or else for them to remain practically unstained while the surrounding dried plasma exhibits markedly basophilic characters. It is to be noted, however, that malaria parasites, spirochetes, etc., remain readily recognisable in spite of such altered staining affinities on the part of the red blood corpuscles.

If the staining is too blue, differentiation may be obtained by washing with 1 to 0.1 per cent. boric acid or monosodium phosphate solution and then with water, the phosphate is the more intense in its action.

Shute's method of using Leishman's stain for thin blood films in the diagnosis of malaria (James). New microscope slides of half white quality are cleaned by dipping into a mixture of 30 parts nitric acid and 70 parts absolute alcohol, and after being wiped dry are stored in absolute alcohol (methylated spirit must not be used at any stage). Before use a slide is wiped dry with a clean cloth.

Preparation and use of stain solution. Pure methyl alcohol ('free from acetone') is used. The pH of the alcohol is tested in a mixture of 1 volume with 3 volumes of distilled water of pH 7.2 (adjusted by adding a saturated solution of lithium carbonate drop by drop to water containing 1/10 volume of 0.01 per cent. phenol red solution). If the pH of the mixture is 7.0 the alcohol is suitable and water of pH 7.2 should be used in staining and washing. If the pH of the mixture is under 7.0, proceed as before, using water adjusted to pH of 7.4, should the tint of the alcohol-water mixture indicate a pH of 7 to 7.2 then distilled water of pH 7.4 should be used in staining and washing. But if the mixture is more acid than this the alcohol should be discarded. To 100 c.c. of the alcohol in a hard (green) stoppered glass bottle previously washed out with the methyl alcohol, add 0.15 gm. of dry stain (Leishman's powder). Shake from time to time in the course of 24 hours, when solution will be nearly complete and the stain ready for use. Place 4 drops of the stain on the blood film and rock for exactly 10 seconds, then add 12 drops of the adjusted distilled water and mix thoroughly, tilting and rocking. Let staining proceed for 30 minutes under a Petri dish for routine identification of parasites. Flush off the stain with a good stream of distilled water. Continue washing for exactly 15 seconds. Older parasites will be overstained but younger

construction

For staining sections a slight modification is necessary. A paraffin section is taken

tageous)—the effect being watched under a low power lens. The blue begins to come out, and the process is allowed to go on till only the nuclei remain blue. The section is then washed with distilled water rapidly dehydrated with alcohol cleared and mounted. If as sometimes happens, the eosin tint be too well marked, it can be lightened by the action of 1 : 7000 solution of caustic soda, this being washed off whenever the desired colour has been attained.

In certain cases, e.g. for the staining of old films or of trypanosomes or Leishmaniae in sections, Leishman recommended an initial treatment of the preparation with serum

Giemsa's stain. According to Giemsa the reddish-blue hue characteristic of the Romanowsky stain is due to the formation of methylene azure, and he has prepared this under the name 'Azur I'. From this, by the addition of an equal part of medicinal methylene blue, he prepared what he called 'Azur II', and from this again by the addition of eosin he prepared 'Azur II-eosin'. The latest formula for the finished stain¹ is as follows: Azur II-eosin, 3 grms.; Azur II, 0.8 gm.; glycerol (chemically pure), 250 grms.; methyl alcohol, 250 grms. This stain keeps well in stoppered bottles (For a method of preparing the stain, see Lillie)

(1) Immediately before use fix films in absolute alcohol for fifteen to thirty minutes, dry with filter-paper. (2) Dilute the stain with distilled water (which must not be acid; it should be boiled for five to ten minutes in a flask to drive off CO_2 and it should be kept stoppered)—one drop of stain to 1 c.c. water (the mixture being gently shaken). (Sometimes the water is made alkaline by the addition of one drop of 1 per cent. potassium carbonate to 20 c.c. water.) (3) Stain for thirty minutes; for spirochaetes a longer period

paper, dry, and mount.

The film, fixed as above or he diluted stain and warmed 1 fresh stain added, the film is again heated; this procedure is repeated four or five times. Finally, the film is washed in water and dried.

Old films containing protozoa tend not after staining and brief, brisk rinsing in water containing a 1:1,000 solution of NaH_2PO_4 i

use the diluted stain.

FIXATION OF TISSUES FOR DEMONSTRATING BACTERIA IN SECTIONS

Good histological fixatives permit of the satisfactory demonstration of bacteria in sections by staining. In general, infected tissues should be fixed as soon as possible, since lytic processes are liable to progress rapidly in these; the pieces of tissue should be small (not over 5 mm. thick), so as to permit rapid penetration of the fixative. In the case of surgically removed tissue, if this is not obviously infected and especially if it contains muscle, it is advisable to allow two to four hours to elapse before fixing it. During this time it is important to keep the material enclosed in gutta-percha or other waterproof fabric, because drying of the surface by contact with absorbent material, such as gauze, or by exposure to air causes damage to the surface.

FIXATIVES

Formol-saline (sodium sulphate 3, sodium chloride 1, formalin 20, water 180 parts). This is a slow-acting fixative and often unsatisfactory for tissues which are to be embedded in paraffin. It is, however, suitable for the preparation of frozen sections (also it allows sufficient restoration of colour to provide a fairly good museum specimen, although inferior for this purpose). Tissues which have been fixed with or without chloral hydrate) be transferred directly days' immersion then be

Formol-corrosive (nine parts 5 per cent. watery HgCl_2 solution to 1 part of commercial unneutralised formalin, this mixture keeps practically indefinitely; the formalin gives a distinctive pink colour and also has a strong odour). This is used in 1:10 to 1:50 dilutions.

formation of granular deposit related to lysing process

¹ It is obtainable commercially.

is liable to occur with any fixative containing formalin, is difficult to remove, but it can be prevented by transferring the tissue after four to six hours in formal-corrosive to 5 per cent aqueous HgCl_2 , in which secondary fixation is allowed to proceed for at least several days—again on wool and well immersed. This double fixation is not necessary for surgically removed tissue unless there has been old hemorrhage, but is highly advisable with post-mortem material. Already formed deposit can be removed in great part if the first portion of absolute alcohol used in the dehydration process is saturated with picric acid. It should be noted that malarial pigment (which is indistinguishable from formalin-deposit in sections) is also soluble in picric-alcohol. Thus tissues from cases of malaria should not be fixed in formal-corrosive. Formal-corrosive is not a good fixative for demonstrating the characteristic changes in the brain in rabies. Tissues from formal-corrosive or mercuric chloride solution should be transferred directly for dehydration to 70 per cent alcohol, the removal of the mercuric deposit by iodine being deferred until the sections are brought into water for staining (p. 776).

For storage and transmission, tissues which have been fixed as above described are removed from the mercuric chloride solution, washed for half an hour in running water and immersed in a mixture of glycerol 3 parts and water 7 parts. After two days remove the tissue from the fluid, surround it with cotton wool which has been soaked in the glycerol-water mixture, and wrap the whole in a sheet of gutta percha tissue, then pack in a strong tin. Before dehydrating the tissue should be washed in water for several hours.

Zenker fixatives (stock solution potassium bichromate 5, sodium sulphate 2, mercuric chloride 10, water 200 parts). In the case of fresh tissue, before use add 1.5 to 5 per cent of glacial acetic acid to the stock solution. The mixture containing acetic acid is a rapid and good fixative but suitable only for small pieces of tissue. Over-fixation must be avoided, after four to six hours the tissue must be transferred to running water and washed for a similar time. It is then dehydrated as usual. With post-mortem material it is advisable to omit acid, because the latter accentuates lysis of the blood, which is likely to be commencing. Bichromate fixatives are chiefly of value for tissues which are to be stained with a Romanowsky mixture. Zenker's solution without the addition either of formalin or acetic acid should be used to fix tissue from cases of malaria, as it does not produce deposits resembling malarial pigment.

For storage and transmission, the fixed tissue after washing in water, may be transferred to glycerol-water and treated as described above.

For the further treatment of fixed tissues with a view to preparing sections, works on histological methods should be consulted.

III

SEROLOGICAL METHODS

METHODS OF MEASURING AND DILUTING SMALL QUANTITIES OF FLUID

Graduated Pipettes. The simplest method is by means of 1 c.c. and 0.1 c.c. pipettes. Each pipette should be graduated in tenths, and should deliver to the end. Much depends on the use of satisfactory pipettes.

Mode of use. A mouthpiece of glass quill tube is attached to the pipette by india-rubber tubing. To obviate the danger of sucking up living cultures into the mouth a small piece of cotton wool may be packed loosely into one end of the india-rubber tube. The mouthpiece is held in one corner of the mouth between the teeth, and the junction



FIG. 223 Use of Pipette

to close it, and continue to keep the tongue in this position until operation (4) is completed, (2) withdraw the nozzle of the pipette from the fluid, and bring it against the side of the vessel above the level of the fluid; (3) while maintaining the nozzle continuously against the vessel-wall make slight pressure on the rubber with the fingers and thumb of the right hand until the level of the fluid in the pipette reaches the 0.05 mark; then do not make any further pressure, (4) then insert the pipette into the vessel into which the fluid is to be delivered (if the amount to be delivered is very small, e.g. 0.01 c.c., pass the nozzle down to within a short distance of the fluid in the tube) and (5) blow out vigorously—while blowing, bring the nozzle into contact with the side of the tube above the level of the contents (The object of bringing the nozzle

at the nozzle as a drop, which would cause a serious error in the measurement of the amount delivered.) Fig. 223 shows the mode of holding the pipette, the reader being in the same position as the operator.

The pipette must be well washed out with saline between successive reagents or successive dilutions of the same reagent. There is the risk, however, when using the same pipette to prepare increasing dilutions, that the calculated dilution is higher than the actual. Accordingly a fresh pipette should be used for each dilution, or traces of very active substances such as toxins or antiserum may be destroyed by boiling, but care must be taken that albuminous matter does not coagulate in the pipette and also that it is cool before use.

Drop Method. Another method of measuring fluids depends on the use of dropping pipettes. When one pipette is used throughout the procedure is simple, and it is accurate when fluids such as serum and saline are concerned. The pipette, which consists of a length of quill tube drawn out to a capillary end, is actuated by an india-rubber teat, the pipette must always be held vertically and the drops must be delivered slowly (at the rate of about 1 drop per second). Before passing from one reagent to another the pipette must be well washed out and either dried with alcohol and ether or rinsed out

with the reagent to be measured by taking up and rejecting the latter several times. When pipettes have to be interchanged, Donald's methods of standardising should be used.

Capillary Pipette Method. The serum is drawn up in a capillary tube (a piece of quill glass tubing drawn out in the flame at one end is convenient for the purpose) and a mark is made at the upper limit of the fluid, the latter then being blown out in a watch glass. Equal amounts of 0.85 per cent salt solution are measured out with the marked tube and added till the required dilution is made. Thorough mixture is effected by drawing up the diluted serum in the quill tube and blowing out again, this being repeated several times. Wright's work should be consulted for his methods of measurement.

AGGLUTINATION

Method of obtaining agglutinating sera. For the identification of organisms by their agglutination reactions artificial antisera of high titre must always be employed (human sera from cases of natural infection should never be used). Well grown rabbits are in general most suited for developing specific antisera—old animals ought to be avoided. Cultures killed by heat are generally effective, as low a temperature as possible being employed, e.g. half to one hour at 56° C. Intravenous injection is as a rule to be preferred, a small dose being given at first ($\frac{1}{10}$ of a killed twenty-four-hours agar slope culture suspended in saline), followed by gradually increasing doses (each being double the preceding one) at intervals of seven to ten days. (Formalin 1:1,000 or other antiseptics may be used to kill the organisms, also a broth culture may be employed, the initial dose being 0.5 c.c.) During the course of immunisation the animal should be weighed frequently. Loss of weight suggests that the dosage is too high, if the animal's health is seriously upset powerful antisera will probably not be obtained. But it must be remembered that some animals respond to the injections by more active production of antibodies than others. In the case of highly toxic organisms, such as Shiga's dysentery bacilli a smaller initial dose is used, e.g. $\frac{1}{10}$ of a culture or their toxicity may be diminished by heating at 80° C. for an hour. Seven to ten days after the second or third dose a specimen of blood may be withdrawn from the ear vein and tested. If the titre is high (2,000 or more—although much higher titres may be obtained with many organisms) the animal may be bled (p. 807).

Serum for the Widal reaction. Blood is obtained and the serum separated (for methods, vide pp. 808, 809).

Serum dilutions may be made (a) by means of a graduated pipette or by the drop method. In this way successive dilutions can be rapidly made in 3 in. by $\frac{1}{2}$ in. test tubes etc. though, his is the best

young, well

are suitable, or killing may be effected by using saline to which 1:1,000 formalin has been added. For methods of distinguishing flagellar and somatic agglutination, vide p. 795. In the case of rough variants, which tend to sediment spontaneously in physiological saline, the reduction of the NaCl content to 0.4 to 0.1 per cent. often yields a stable suspension. With streptococci the nature of the culture medium on which the organisms have grown is important, various devices may have to be tried in order to obtain stable suspensions (p. 162). Shaking the suspension in a vessel with glass beads may help to render it homogeneous.

Naked-eye method of test for agglutination is much preferable to the microscopic, as the results with the former are more accurate. Equal parts of diluted serum and of bacterial suspension are mixed and placed in an agglutination tube or a narrow test tube ($\frac{1}{8}$ in. diameter). Keep in the upright position at room temperature for twenty-four hours at 37° C. for four hours, or at 55° C. in a water-bath for two hours (the convection

currents set up when the tubes are only partially immersed hasten the appearance of flocculation). The results are best read with the naked eye by holding the tubes against the light to observe the serum. The serum which produces agglutination is 1 : 8,000, this is expressed by saying that 8,000 is the 'titre' of the serum.

The details of this procedure are as follows in the case of testing a patient's serum with *B. typhosus*. A series of small (3 in. by $\frac{1}{2}$ in.) test-tubes, e.g. six, is set up. Into each of the tubes with the exception of No. 1, 0.4 c.c. saline is measured. Then to 0.1 c.c. serum there is added 1.4 c.c. saline, thus making a 1 in 15 dilution. Of this dilution 0.4 c.c. is added to tubes Nos. 1 and 2, and the contents of the latter are mixed by drawing the contents of tube No. 2 into tube No. 3, and so on, until tube No. 5 contains 0.4 c.c. of the original dilution. Tube No. 6 contains 0.4 c.c. of the original serum. Each tube and the

obtained,
1 : 2,000, 1

Microscopic form of test. Equal quantities of the diluted serum and the bacterial suspension are mixed under the $\frac{1}{2}$ in. objective. A well will be found very suitable for the original dilutions.

Flocculation on a slide is a useful method for preliminary observations, e.g. for testing suspicious colonies from plate cultures of faeces in order to determine which should be

tion occurs, obvious granularity to the naked eye develops. Since different cultures of the same organisms (and their agglutinability by the rich cultures. The organism ordinary veal peptone broth shaken, and there is added to it 0.1 per cent. of commercial formalin, it is again shaken and placed at once in a cool chamber at about 2° C in the dark. The shaking is repeated at intervals for four to six hours. At the end of three or

In testing a patient's serum a series of dilutions and a control without serum are prepared in agglutination tubes as follows.

	TUBE				
	1	2	3	4	5
drops of saline	0	5	8	9	10
1/10 serum	10	5	2	1	0
standard culture	15	15	15	15	15
final dilution =	1/25	1/60	1/125	1/250	Control

represents flocculi just visible to the naked eye. In the case of the enteric organisms

Agglutinable cultures and control tubes showing standard agglutination for enteric and dysentery bacilli etc. are obtainable from the Central Public Health Laboratory, Colindale Avenue, London, N W 9, which also issues directions for their use.

reference should be made to the Medical Research Council's Special Report Series, No 51)

Flagellar and Somatic Agglutination. Motile bacilli of the normal type of culture of the coli-typhoid group, etc., may be agglutinated by antisera acting on the H antigen which is present chiefly in the flagella. The result is rapid formation of large flakes which settle quickly to form a voluminous sediment. On the other hand, antisera which act only on the somatic (O) antigen lead to slow formation of small closely packed granules. The latter form of agglutination is the only one shown by non-flagellate organisms, e.g. dysentery bacilli¹

Strains of organisms rich in the respective antigens are selected (*B. typhosus* strain 901 H and O variants, etc., obtainable from the National Collection of Type Cultures), and twenty-four-hours' growths are employed. To promote the formation of H antigen, the agar medium used should be prepared from fresh meat (not meat extract), react neutral, have abundant water of condensation, and be sterilised at 100° C., the state of the cultures must be controlled from time to time (a) with known anti-H and anti-O sera from rabbits, to determine the presence of the respective antigens, and (b) with hypertonic (3.4 and 6.8 per cent) NaCl solution, in which the organisms should form a permanent suspension; sedimentation would indicate change to the rough state, which is unsuitable.

For eliciting flagellar (H) agglutination, suspensions of living organisms are best, but failing these, suspensions prepared with phenol or formalin may be used (*vide supra*).

For the detection of somatic (O) agglutination in the case of an organism which

ring. After the mixture has stood for twelve to twenty-four hours at 37° C., in a capped

The titre of the serum may then be determined in the usual way by the naked-eye method. In the case of living suspensions the results are read with a $\times 10$ magnifier after two hours at 37° C., and again after sixteen to eighteen hours further at room temperature, since small flake agglutination occurs slowly. With alcohol-treated suspensions twenty-four hours at 52° C. should be allowed.

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m or where the result is negative, the
ll circular layer with a sharply defined

margin

Agglutinin Absorption

diluted ten times) an equal volume of a thick suspension (the organisms on one agar slope being used for 2 c.c. of diluted serum), allowing the mixture to stand at 37° C. for two or

¹ See note, p. 794.

three hours, or in the ice-chest overnight, and then separating the bacteria with the centrifuge. The supernatant clear fluid is now pipetted off, and its agglutinating properties studied on the other members of the bacterial group and also, by way of control, on the organism used for absorbing it, in order to make sure that all agglutinins for the latter have been removed. Should this control show that absorption of agglutinins for the homologous organisms is incomplete, then the serum should be absorbed again with a fresh portion of culture.

Similarly, agglutinin for particular antigenic constituents of an organism may be obtained by the absorption method. Thus in the case of *B. typhosus* an agglutinin for somatic antigen may be prepared from an antiserum containing

COMPLEMENT-FIXATION REACTION OF BORDET AND GENGOU

The principle of the reaction

The reaction is carried out on this analogy (*vide infra*). A further use of the reaction is to identify an antigen (an organism, or a protein solution such as serum, etc.) by showing that in the presence of a given immune-body it causes fixation of complement.

Guinea-pig's Complement. The animal is bled by severing the neck vessels over a large glass funnel (6 in. diameter) which dips into a measuring cylinder—the glass-ware is sterilised beforehand by heat, and when cool is rinsed with normal saline. After the blood has coagulated, the clot is removed, and the serum is separated by centrifuging in order to aid separation of the clot. The serum is then separated from the clot by centrifuging. The serum is then separated from the clot by centrifuging. The serum is then separated from the clot by centrifuging.

when kept at room temperature, the serum should be separated from the clotted blood which has been kept in the ice-chest for eighteen hours; it is then ready for use. The complement can be preserved for several months in the frozen state at a temperature below -10°C . Complement which has been dried from the frozen state *in vacuo* (*vide p 770*), also furnishes a stable reagent which keeps well; before use it is dissolved in sufficient distilled water to make up the original volume.¹ Complement may also be preserved by chemical methods, e.g. addition to the serum of an equal volume of a solution

consisting of boric acid, sodium borate, sorbitol, and sodium azide in saturated NaCl solution. In all cases the strength of the complementing serum must be titrated at the commencement of every experiment in which it is employed (*vide p 94*).

SERUM DIAGNOSIS OF SYPHILIS

The Wassermann Reaction (Wassermann, Neisser and Bruck)

The Wassermann reaction depends on the phenomenon of complement fixation of Bordet and Gengou (pp 94, 486). It must be noted that the substance in the syphilitic serum which leads to the fixation of complement may vary greatly in amount in different cases, and in the same case at different times, especially under the influence of anti-syphilitic treatment with salvarsan drugs, etc. Accordingly, it is not possible to state absolutely the quantity of complement which must be fixed in order to give a positive result. Manifestly there will be cases where the amount fixed is just under any standard adopted and these, which are to be regarded as weakly positive, or doubtful, will be missed unless the reaction is carried out with several different amounts of complement. Moreover, the amount of complement, as estimated by the hæmolytic dose, varies considerably in different samples of guinea-pig serum, also the complement in different specimens of such serum varies in its capacity for being fixed (deviability). It is accordingly necessary for satisfactory results to estimate the hæmolytic dose of the guinea-pig's serum, and to make a series of mixtures, each containing the same amounts of serum and of antigen, but with a different number of doses of complement in each tube. By such

¹ Dried complement can be obtained commercially

a quantitative method one can ascertain the number of doses of complement fixed in each case. As controls, the effect on doses of complement of the antigen alone and of the serum alone should be tested, also, known negative and positive sera should be tested at the same time (the latter should include one which gives only a weak reaction).

Quantitative method. In order to carry out the test, we require (a) the patient's serum previously heated at 55° C., (b) an alcoholic tissue extract (that of human or ox heart, along with cholesterol being most widely used) (c) the fresh serum of a guinea pig to act as complement, and (d) sensitised corpuscles i.e. a suspension of washed sheep or ox corpuscles to which have been added five doses of immune-body (*vide p. 94*). Three tubes with different doses of complement will be sufficient for routine examination. Also, along with each series of tests the necessary controls must be included (*vide infra*).

OUTLINE OF THE TEST. To each of the three tubes add 0.5 c.c. of *diluted antigen* (*vide infra*) and 0.05 c.c. of the serum to be tested which has been heated for half an hour at 55° C. shortly before the test.

Add to the three tubes respectively two, four, and six doses of complement—the dose being that for 0.5 c.c. of sensitised corpuscles.

Place the tubes in the incubator for an hour and a half at 37° C.

Then to each add 0.5 c.c. of suspension of corpuscles sensitised with five doses of immune-body, and place again in the incubator for an hour and a quarter. Place the tubes aside at the room temperature till the non lysed corpuscles have sedimented—at least for half an hour—and then read the results.

Controls should be made in each series of tests as follows: tubes containing the stated amount of antigen along with 2, 4, 6 doses of complement, and for each serum under test one containing the stated amount of heated serum in 0.5 c.c. saline along with two doses of complement, to all these 0.5 c.c. of sensitised corpuscles is added after incubation for an hour and a half. It is also essential to put up tests with a known negative (preferably a pooled specimen of at least six sera) and positive sera including a weakly positive serum—in routine work it is convenient to keep such sera from previous tests, they are best preserved by freezing, but if sterile they will keep for a few days in the ice-chest.

Antigen. Various antigens have been used but the following gives very satisfactory results. Human heart muscle from the left ventricle (the cause of death is stated to be immaterial) is freed from visible fat, minced finely, and ground for a minute with absolute alcohol (1 gm. of minced muscle to 9 c.c. of alcohol) in a mortar with clean sand. The mixture is shaken in a mechanical shaker for one and a half hours and then filtered. This constitutes heart extract which keeps at cool room temperature for six months at least. Ox heart extract may be prepared similarly.

Preparation of diluted antigen. 1.5 volumes of heart extract are mixed with 1 volume of 1 per cent. cholesterol in absolute alcohol. 1 volume of this solution is mixed rapidly with 29 volumes of normal saline. It is advisable in the case of each specimen of extract to test various dilutions of it with known syphilitic sera and find the dilution which gives greatest fixation of complement and then to use this dilution in subsequent tests.

DETAILS IN PERFORMING THE TEST

Reagents¹:

(a) *Patient's serum* heated at 55° C. for thirty minutes before use (unheated serum from non-syphilitics may react positively).

(b) *Antigen dilution.* An alcoholic tissue extract (that of human or of ox heart + cholesterol) diluted with saline as described above.

(c) *Complement.* Mixed fresh serum of at least two guinea-pigs (*vide p. 796*). The blood should be drawn and kept overnight (eighteen hours) in the ice-chest, the serum is then separated for use. The complement of serum obtained too short a time after bleeding may be unsuitable as it tends to be excessively deviable—see also p. 95. As a guide to the amounts of complement to be used the W.H.D. is estimated immediately before carrying out the Wassermann reaction as follows. Prepare a 1 in 4 dilution

¹ Use heavy 3 in. by $\frac{1}{2}$ in. test tubes well cleaned and finally thoroughly rinsed in distilled water to remove traces of acid alkali, or soap and then dried in the hot air oven. Convenient tube racks have top and ends formed by a single piece of No. 22 B.W. gauge zinc or aluminium the top measures 11 in. by 2 $\frac{1}{2}$ in. and the height of the ends is 2 $\frac{1}{2}$ in. Along each side of the top a strip of the metal $\frac{1}{2}$ in. deep is turned down vertically. There are three sets of twelve equally spaced perforations in the top, each $\frac{1}{8}$ in. in diameter. The base is of hard wood $\frac{1}{2}$ in. thick, in which there are cupped depressions $\frac{1}{2}$ in. deep in the centre and $\frac{1}{8}$ in. in diameter, placed vertically under the perforations in the top. The foot of the base is flush with the metal ends, to which it is secured by screws.

of complement (0.3 c.c. saline plus 0.1 c.c. guinea-pig's serum); of this add to four tubes each containing 0.5 c.c. sensitised red cell suspension plus 0.5 c.c. saline, 0.01, 0.02, 0.03, and 0.04 c.c. respectively. Mix the contents well by shaking and keep the tubes at 37° C. for one hour—shaking at fifteen minute intervals. Take as the M.H.D. the smallest amount of complement which causes practically complete lysis; it is usually that in the second or third tube, i.e. 0.005 or 0.0075 c.c. of undiluted complement.

(d) *Sensitised red cells.* Defibrinated or oxalated blood of sheep¹ or ox is freed from serum by mixing with 3 or 4 volumes of 0.85 per cent NaCl solution, centrifuging, removing the supernatant fluid, adding fresh saline, etc., the procedure being repeated three times. The amount of blood used is 97 c.c. of body

infrequently with sheep corpuscles, lysis is interfered with. To avoid this the red cell suspension should be sensitised about ten minutes before use. The dose of immune-body remains fairly constant when the serum is kept at 5° C., also it does not as a rule differ much for different batches of the homologous blood. Accordingly, it is not necessary to estimate the dose for each set of tests.

Add reagents in the order shown:

	Tube No.	1	2	3	Serum Control
					4
Saline, c.c.		—	—	—	0.5
Antigen dilution, c.c.		0.5	0.5	0.5	—
Patient's serum (55° C.), c.c.		0.05	0.05	0.05	0.05
Complement M.H.D.		2	4	6	2

Mix well the contents of each tube; incubate at 37° C. for one and a half hours, shaking every thirty minutes; then add to each tube 0.5 c.c. sensitised sheep (or ox) red cells, incubate again for one and a quarter hours at 37° C., shaking every fifteen minutes.

Controls for each Series of Tests.

	Tube No.	1	2	3
(A) <i>Antigen Control.</i>				
Antigen dilution, c.c.		0.5	0.5	0.5
Complement M.H.D.		2	4	6

	Tube No.	1	2	3	4
(B) <i>Complement Control</i>					
Saline, c.c.		0.5	0.5	0.5	0.5
Complement (diluted 1/4 with saline), c.c.		0.01	0.02	0.03	0.04

(C) In addition, a known pooled negative and a known weakly positive serum are always tested (*vide supra*).

The controls are incubated, etc., along with the main test. Serum and antigen controls show that these reagents separately have only minimal inhibiting effect upon complement. The complement control shows that the complement is active.

Read results after the tubes have stood at room temperature to allow any unlysed red corpuscles to sediment (*vide infra*).

Cerebro-spinal Fluid is tested in the same way as serum, except that it is not heated beforehand. 1 volume of the alcoholic antigen is diluted quickly with 29 volumes of the fluid, and 0.5 c.c. of this mixture is measured into each tube. In the control tube, instead of serum and saline, 0.5 c.c. of cerebro-spinal fluid is used.

Reading of Results. It will usually happen that the test with the antigen alone, at the serum alone, and also that with the mixture of antigen and the negative control

plate lysis of the negative control serum is negative (b) A serum which produces a and a half times to twice as much complement as does the negative control to produce a given degree of lysis is *suspicious*—recorded as '±' or '±' (c) A serum which fixes more complement than is specified under (b), but which shows complete lysis with the

¹ Such blood and the corresponding immune body are obtainable commercially. It is received direct from the animal's neck with a sterile wire whisk. If one-tenth per cent of proflavine in normal saline is added 1 week in the ice-chest.

highest amount of complement, is a *weak positive* (d) A serum which shows no lysis in any tube is a *strong positive*, i.e. one which fixes upwards of five doses of complement more than the sum of the amounts inhibited by the antigen and the patient's serum separately.

given is to be preferred. Occasionally where the usual amount of patient's serum gives only a doubtful positive result, increasing the amount to 0.1 c.c. or 0.2 c.c., may lead to more marked fixation of complement. Altering the dilution of antigen is stated to have a similar effect sometimes. Some workers prefer to allow serum, antigen and complement to react for a longer time at ice-chest temperature, instead of at 37° C.

Other methods of performing the test are described by Wyler, Eagle, Richardson; and others.

Flocculation Reactions

The occurrence of precipitates when syphilitic sera were mixed with lecithin and other colloids was occasionally observed soon after the discovery of the Wassermann reaction, but reliable forms of this test were devised considerably later.

SACHS-GEORGI METHOD Each patient's serum, which should be freshly taken, is heated at 55° C. for half an hour and 0.4 c.c. of each of the following dilutions is prepared in six 3 in. by $\frac{1}{2}$ in. test tubes—1, 2, 1, 4, 1, 8, 1, 16, 1, 32, 1, 64—and a further tube receives 0.4 c.c. saline (this acts as a control of the antigen emulsion for the series of tests). Then to each tube 0.2 c.c. of the antigen emulsion is added. When the optimum dilution of the antigen in the test is 1:18 a 1:6 emulsion is used. The latter is prepared by mixing rapidly equal quantities of the undiluted antigen and saline solution, allowing the mixture to stand for ten minutes and then adding sufficient saline to give the required dilution.

tubes (p
temperatu
eight hours

they are brightly lit without the rays of the lamp reaching the eyes directly. Strongly reacting sera may cause flocculation in the highest dilutions, with weak sera flocculation may be present only in the tube with the highest amount of serum. The appearances resemble those in bacterial sedimentation. Zone phenomena sometimes occur in which flocculation is less with the higher concentrations of serum than with lower. Known negative and positive sera should be included in every series of tests. In the antigen control and in tests with negative sera no flocculation occurs, the contents of the tubes being homogeneous and slightly opalescent.

Antigen This is prepared as follows: 20 grms. of sheep's heart muscle, freed from gross fat and fibrous tissue, are ground with sand in a mortar and extracted for four days at room temperature with 100 c.c. of 96 per cent alcohol. The extract is filtered and 0.25 gm. cholesterol is added and the mixture is kept at 37° C. for some days. It is then allowed to stand at room temperature for a day and filtered. This solution keeps indefinitely. In order to standardise a new antigen it must be compared, as regards the sensitiveness of different dilutions, with the optimum dilution of an antigen whose behaviour has been previously tested. For this purpose emulsions are prepared from 1:1 cholesterol-extract saline mixture made as described above by adding to a fixed amount

new antigen which is at least as sensitive to positive sera as the optimum of the old, and which does not flocculate either alone or with negative sera, is selected as the optimum dilution. Krishnan has described in further detail methods of standardising the antigen. Difficulties of this test are the preparation of a sensitive antigen and that the sensitiveness of the antigen emulsion tends to vary in an unpredictable way, e.g. on occasions the emulsion will be flocculated by the control negative sera.

MEINICKE'S METHOD—FORD ROBERTSON-COLQUHOUN MODIFICATION In this procedure, an extract of ox heart together with tolu balsam forms the basis of the antigen.

¹ Antigen for the Sachs Georgi test may be obtained commercially

it requires to be carefully standardised and it is advisable to use a centrally controlled preparation.¹ The patients' sera are used unheated. A stock of NaCl ('A. R.') solutions of 3 per cent, 1.5 per cent, and 1 per cent. strength are kept for diluting the antigen.

Serum. With a clean 1 c.c. pipette graduated to the tip measure into three thick-walled 6 cm. by 0.7 cm. test-tubes 0.3, 0.2, and 0.1 c.c. of patient's serum.

Antigen dilutions.—for testing twenty sera Into each of three 6 in. by $\frac{1}{2}$ in. test tubes (A, B, C) measure 1 c.c. of antigen solution and into other three 10 c.c. respectively of 3 per cent (A¹), 1.5 per cent. (B¹), and 1 per cent. (C¹) saline; place the latter three tubes in a water-bath at 56° C. together with a fourth tube containing 10 c.c. of water and a thermometer. When this registers 56° C. place the tubes of antigen solution in the water-bath and after two minutes pour each 10 c.c. of saline rapidly into the corresponding tube of antigen and mix thoroughly by pouring backwards and forwards (thus, pour the antigen-saline mixture from tube A into A¹ and finally return the contents to tube A)—care must be taken that the outsides of the tubes are dry, so that no water from the bath gets into the antigen solution. The antigen dilutions, which should appear homogeneous, white and opaque, are then ready for pipetting into the sera (they should not be returned to the water-bath) and must be added at once, since on standing the reactivity of the emulsion diminishes. A dry 5 c.c. graduated pipette is used for this purpose, a separate one being taken for each antigen dilution. The tubes containing 0.3, 0.2, and 0.1 c.c. of serum receive respectively 0.5 c.c. of antigen dilutions A, B, and C. Each tube is well shaken, so as to mix its contents thoroughly. As a control, a test of a known negative serum is included in the series. All the tubes are then placed at about 20° C. in an incubator or a cupboard free from draught for sixteen to twenty hours, when the results are read.

Reading the results. A bulky deposit at the foot of the column of fluid along with complete clearing of the supernatant is recorded as +; if the supernatant is hazy it is read as + tr h (trace haze) or + h (haze), according to the degree of opacity. When the supernatant is opaque with only a small disk or button of deposit at the foot the reading is negative (—).

The result is returned as *strong positive* when tube C shows complete clearing and tubes B and A show partial or no clearing, *positive* when all three tubes or at least A and B show complete clearing, *weak positive* when only tube A shows complete clearing.

As in the case of the Wassermann reaction, a weak positive result should not by itself be regarded as of diagnostic significance.

A comparison of the results of the three reactions carried out by the above methods has been made by Colquhoun, Kyles and Rannie.

Other methods of performing the flocculation test are described by Mackie and McCartney, Kahn, etc. See also League of Nations Publications, *Ten Dis Informn.*, Supplement No. 9, Washington, 1930.

OPSONIC ACTION

Opsonic action was demonstrated by Leishman by a simple method. Equal quantities of blood and of a dilute suspension of the organism to be tested are mixed together, a small drop of the mixture is placed on a glass slide and covered with a cover-glass, the preparation is placed in the incubator at 37° C. for fifteen minutes. The cover-glass is then slipped off and the film on the slide stained by Leishman's method. The number of bacteria present in, say, fifty polymorphonuclear cells successively examined is determined, and an average struck. Since phagocytosis depends upon certain substances in the serum to which Wright gave the name *opsonins*, he elaborated the following method by which its degree could be estimated.

(1) **Preparation of bacterial suspension.** In the case of organisms such as the pyogenic cocci, some of a twenty-four-hours' living culture on an agar slope is rubbed up with 0.85 per cent saline so as to obtain a suspension consisting of single bacterial cells. With certain organisms, e.g. streptococci in chains, a good deal of trituration may be necessary, and often centrifuging must be practised, for the removal of clumps. Only by experience can a knowledge be gained of the amount of culture to be used in the first instance, but the resultant suspension usually should exhibit only faint cloudiness to the naked eye.

¹ This may be obtained from the West of Scotland Neuro-psychiatric Research Institute, 10 Shelley Road, Glasgow, W 2.

the deposit washed with saline. In the case of the tubercle bacillus, a seven- to ten-day culture is allowed to reach about the standard turbidity, collected on a filter washed with

the bulk of about 1 c.c. This is centrifuged and the supernatant suspension pipetted off and diluted to the necessary degree

then centrifuged, and when the corpuscles are separated the supernatant fluid is removed, fresh saline is substituted, and the centrifuging repeated. A second washing with saline is practised, the supernatant fluid removed, and the greyish surface layer of blood, which is rich in leucocytes, removed by a fine pipette. The leucocytes may be thoroughly mixed by drawing up in a fine pipette and blowing out again, this being repeated several times.

(3) *Preparation of the sera* Each sample of serum is prepared by the methods described on p. 809. In every case serum from a normal individual should be used as a control.

The suspension, leucocytes, and serum being thus prepared, an equal quantity of each is taken by a small capillary pipette, and a thorough mixture is made in the usual way. A small portion of the mixture is taken up in a capillary tube, and its ends are sealed by heat, care being taken that the contents are not over-heated. The tube is then placed in the incubator at 37° C. for fifteen minutes—it is an advantage to rotate the tubes

constitutes the *opsonic index*—that of normal serum being reckoned as unity.

Thermostable opsonins These are estimated similarly, except that the serum is heated beforehand, say at 55° C. for a half to one hour.

In the case of organisms such as those of the coli-typhoid group and cholera which are susceptible to bacteriolysis, it may be necessary to heat the sera at 55° C. This destroys any complement present and prevents bacteriolysis occurring.

Several modifications of Wright's technique have been suggested (*vide* p. 99).

PREPARATION OF VACCINES: INOCULATION OF ANIMALS: OBTAINING OF BLOOD, PATHOLOGICAL MATERIALS ETC., FOR EXAMINATION.

PREPARATION OF VACCINES¹

The general procedure is to prepare a sterile suspension of dead bacteria. In certain cases the bacteria are subjected to disintegrating processes before being used, but most frequently vaccines simply contain killed bacterial cells. Recent investigations on bacterial variation have shown that the immunising properties of vaccines may depend greatly on the state of the culture from which they are prepared. In many instances recently isolated virulent cultures yield the most effective products; this is well exemplified in

ingly,
will p
conferring protection against the corresponding infections. The special methods adopted in preparing vaccines are described in the chapters on the various diseases. The methods described here apply chiefly to the preparation of 'autogenous' vaccines, i.e. those derived from cultures isolated from individual patients. The dosage of a vaccine is con

a higher temperature must be employed. It is probable that the lower temperature at which the contained bacteria are killed the more efficient is the resulting vaccine. The success of the sterilisation must be tested by transferring some of the heated vaccine to a suitable medium and incubating for forty-eight hours. Thereafter phenol is added to make 0.5 per cent. Appropriate doses are then with all aseptic precautions, measured by means of sterile graduated pipettes, and placed in glass ampoules (bulbs drawn out to a capillary tube at one end). Sufficient 0.5 per cent. phenol in sterile saline is added to make the contents of the ampoule up to about 1 c.c. The ampoules when charged are sealed, i.e. the necks are rendered homogeneous by shaking, the sealed end is broken off, fairly deeply distributing the vaccine is in larger amounts in vaccine bottles, thick rubber cap.² When a dose of the vaccine is required the bottle is well shaken, the cap is sterilised, e.g. with strong lysol, it is punctured with a sterile hypodermic needle attached to a sterile syringe and by drawing up the piston the dose is taken into the syringe.

before they are used, by the following
than 0.1 per cent. of the total
and should in no case be less
much that any phenolic anti-
One-half of the sample
septic present in the sample is diluted to 1:1000
is used for aerobic culture test and the other half for anaerobic

In the case of the aerobic culture, the medium should consist either of meat extract
vaccine is prepared specially for the use of
qualified medical practitioner or, at the latter's request, by another person
² Berry's pattern is made by Britton, Malcolm & Co., 38 Southwark Bridge Road,
London, S.E.1.

³ These tests are specified by the Therapeutic Substances Regulations, in the case of vaccines manufactured for sale under licence

to which 1 per cent of peptone has been added or of an equivalent prepared by tryptic digestion of muscle. After the final sterilisation the pH of the medium should be 7.2 to 7.8.

The medium used for testing for anaerobic organisms should consist of a similar broth with the addition of heat-coagulated muscle sufficient to occupy a depth of not less than 1 cm. at the foot of the tube, the final pH should be 7.2 to 7.8. Before inoculation the anaerobic medium should be heated to 100° C. for a period sufficient to free it completely from oxygen, then cooled to 37° C. or lower.

The inoculated tubes should be kept at 37° C. for five days before the results are read.

Methods of Counting the Bacteria in Dead Cultures. *Hæmocytometer method.* A sufficiently accurate enumeration of the bacteria in a suspension can usually be made by counting a suitably diluted sample on a hæmocytometer slide. 0.1 c.c. of suspension is diluted 1:20 by adding 1.8 c.c. saline plus 0.1 c.c. centrifuged methylene blue and two loopfuls of formalin. A drop of this mixture is placed on the hæmocytometer stage and the number of organisms in twenty to fifty small squares counted. The average number per square of $\frac{1}{40}$ sq. mm. area $\times 80 \times 10^4$ = the number of organisms per c.c. of

assisted by reading printed letters through the suspensions. Permanent standards for comparison may be made by preparing suspensions of inorganic substances such as barium sulphate. Brown has elaborated this method, and Cunningham and Timothy have supplied numerical equivalents of the opacity standards for various organisms.¹

INOCULATION OF ANIMALS

The animals generally chosen for inoculation are mice, rats, guinea-pigs, rabbits, and pigeons. Care must be taken especially in drawing conclusions from isolated experiments on rabbits, as they are very liable to suffer from intercurrent infections. It must be remembered that different animal species exhibit marked variations in susceptibility to different infections, therefore in examining material for a particular organism one chooses a susceptible origin may be chosen.

should have been kept in the laboratory for some days. In the case of the mouse and rat the variety must be carefully noted, as there are differences in susceptibility between the wild and tame varieties, and between the white and brown varieties of the latter and even between different strains. Larger animals are expensive and require to be kept under special conditions, while dogs are, as a rule, not suited for experimental inoculation. For certain infections particular species of monkeys are required.

Most inoculations are performed by means of a hypodermic syringe. The best variety is that of the glass-metal 'Record' type furnished with needles of platinum-iridium or stainless steel of various bores. Before use, the syringe should be taken apart, placed in cold water, and sterilised by boiling for at least five minutes, when cool the parts should be assembled with the aid of sterile forceps. 'Record' syringes are damaged by dry heat but the 'Balco' glass metal syringe² can be sterilised in the assembled state either by boiling or in the autoclave or hot-air oven. 'All-glass' syringes may be sterilised and kept ready for use. The assembled syringe, the plunger of which has been

¹ Burroughs Wellcome, London, supply a series of standard opacity tubes for the purpose, along with explanatory tables.

² Experiments on animals cannot be performed in Britain without a licence granted by the Home Secretary. According to the nature of the experiments and the species of animals employed various certificates are also required. Information on the keeping of animals is contained in *The UFAW Handbook on the Care and Management of Laboratory Animals*, edited by Worden, London, 1947.

³ Supplied by S. Maw, Son and Sons, Ltd., Aldersgate House, New Barnet, Herts.

The materials used for inoculation are cultures, suspensions of animal tissues, fluids, etc. If the bacteria already are in a fluid there is no difficulty. The syringe is most conveniently filled out of a shallow conical test-glass, which ought previously to have been covered with kraft paper and sterilised, or a Petri dish may be used. If organisms growing on the surface of a solid medium are to be used for inoculation, either some of the culture is scraped off and suspended in sterile broth or gelatin-Locke solution, or a small amount of sterile fluid is poured on the growth, and the latter emulsified with the looped wire. Suspensions of bacteria in saline should be used without delay, as some organisms soon die in it, e.g. gonococci or pneumococci. If a solid organ is used for inoculation, it ought to be first cut into small pieces and rubbed up in a mortar with sand and a little distilled water with aseptic precautions. If a suspension is allowed to stand in a conical glass for a few minutes gross particles settle to the foot or they may be centrifuged out at a low speed in a covered container.

The methods of inoculation generally used are: (1) scarification of the skin; (2) intracutaneous injection; (3) subcutaneous injection; (4) intraperitoneal injection; (5) intravenous injection; (6) injections into special regions, such as the anterior chamber of the eye, the cardiac chambers, the substance of the testis, brain, etc. Of these (2), (3),

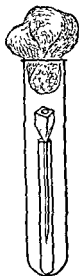
(4), and (5) are most frequently used. The animals should be held by a trained assistant. When an anæsthetic is to be administered to a small animal, this is conveniently done by placing the animal, along with a piece of cotton wool soaked in ether, under a bell-jar or inverted glass beaker of suitable size. Another method, e.g. as a preliminary to intratesticular inoculation in rabbits, is to inject subcutaneously, three-quarters of an hour beforehand, a dose of 1.5 grms. of urethane per kilo of body weight, and to keep the animal warm.

Scarification. The skin of the abdomen is shaved and cleansed with alcohol, or clipped closely and depilated by applying a fresh 5 per cent. solution of sodium sulphide. After one minute the solution is mopped off, and, if necessary, the treatment is repeated until the area is bare (irritation of the skin will result if the application of the solution is unduly prolonged). Finally, the skin is washed well with water and then with spirit and an hour allowed to elapse before inoculation. Then several parallel scratches are made in the skin just sufficiently deep to draw blood, and the infective material is rubbed in with a platinum loop or the side of a scalpel. The disadvantage of this method is that the inoculation is easily contaminated.

Intracutaneous injection is used to test for a local inflammatory reaction, e.g. in order to ascertain the virulence of diphtheria bacilli or to determine sensitiveness to tuberculin. A syringe with a short fine needle (I S W G 25 or 26) is used. With animals such as the guinea-pig, light-coloured or white specimens are chosen, since the results are more easily read. The areas to be injected (preferably

on the abdomen) are first of all clipped and then depilated (*vide supra*). The skin, after being folded into a fold with the thumb pointing towards the entrance into the lax skin, the needle is introduced; a few gentle strokes are given, these

FIG. 224 Method of storing syringe needle. (After Med Res Council War Memo. No 15, 1915.)



on the abdomen) are first of all clipped and then depilated (*vide supra*). The skin, after being folded into a fold with the thumb pointing towards the entrance into the lax skin, the needle is introduced; a few gentle strokes are given, these

should be checked in a report

and the skin

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sinus forming in the connective tissue

may be passed as far as possible into the connective tissue before the inoculation. The wound is sealed with collodion. In the case of mice and tame rats, an assistant holds the animal by the tail and the skin at the nape of the neck.

The hair over the lower part of the abdomen is cut, and the skin is then pinched up between the thumb and forefinger.

Intravenous injection In the rabbit the vein usually chosen is one of the auricular veins. The part has the hair removed, the skin is cleansed, and the vein made prominent by pressing on it between the point of inoculation and the heart. The needle is then passed obliquely into the vein, and the fluid injected. That it has perforated the vessel will be shown by the escape of a little blood, and that the injection has taken place into the lumen of the vessel will be known by the absence of any swelling in the surrounding subcutaneous tissues. In mice and rats a very fine short needle, as for intracutaneous

a linear incision about 0.25 in. long is made parallel to it through the overlying skin and the subcutaneous tissue is then pushed aside with pointed forceps.

Subdural injection in the rabbit The animal is anaesthetised with ether, and the hair between the eyes is shaved, the bare area is then cleansed. A sagittal incision about 1 in. long is made $\frac{1}{2}$ to $\frac{1}{4}$ in. to one side of the mid-line, with its centre opposite the middle of the eyes. The mesial flap is pulled aside and a small hole sufficient to admit a hypodermic needle is drilled 2 mm. to the side of the point where a line joining the outer canthi of the eyes crosses the mid-line of the skull. (A small wedge-shaped, blunt-pointed drill with a cutting edge should be used in order to avoid opening the dura and damaging the brain.) When the dura is exposed a fine needle can be passed horizontally into the subdural space for about $\frac{1}{4}$ in., 0.2 to 0.4 c.c. may be injected slowly. The skin flaps are sutured, and sealed with collodion. For cleansing the skin before incising, painting with a mixture of brilliant green and crystal violet, 0.5 gm. each, dissolved in 100 c.c. absolute alcohol, is most effective, the area is ready when the alcohol has evaporated.

Intracerebral injection in the rabbit A median longitudinal incision is made through the prepared skin over the vertex, the periosteum is then similarly incised, but slightly to one side of the mid-line, and pushed aside to expose the bone. A hole is then drilled, through which a fine hypodermic needle is passed into the brain to a depth of 3 mm., 0.4 c.c. may be injected. On withdrawing the needle the periosteum is at once brought over the hole and the skin is sutured. If it is desired to make the injection into the occipital lobe, the hole is drilled at a point 2 mm. lateral to the sagittal suture and 1.5 mm. anterior to the lambdoidal suture.

In mice intracerebral injection is made as follows: the skin over the vertex is depilated and cleansed, and, under an anaesthetic, a fine needle is passed for about 3 mm. through the bone slightly behind and to the side of the vertex. about 0.05 c.c. may be injected.

Intraspinal injection in the rabbit can be made through the space between the seventh lumbar and first sacral vertebrae, the spine of the former lies in a line with the iliac crests.

Other operative procedures in special regions of the body are carried out with the usual aseptic precautions.

After inoculation, the animals ought to be kept in metal cages, which can be thoroughly disinfected subsequently. Preferably they should open from above, otherwise infective material may be scratched out of the cage by the animal (see Miles and Mitchell). The cages can be sterilised by boiling in a special steriliser. The general condition of the animal is to be observed, and in any experiment in which the animal survives for some time it should be weighed at regular intervals, e.g. weekly. The temperature is usually taken *per rectum*. An ordinary clinical thermometer is smeared with vaseline, and the bulb inserted just within the sphincter, where it is allowed to remain for a minute, it is then pushed well into the rectum for five minutes. If this precaution be not adopted a reflex contraction of the vessels may take place, which is likely to vitiate the result by giving too low a reading. The normal rectal temperature of the rabbit is on the average 102.4° F., but marked variations occur, and up to 104° F. is not a significant increase, that of the normal guinea-pig may be 99.6 to 102° F. Mice are kept singly in cylindrical glass jars measuring 9 in. by 5½ in. The cover for a jar consists of a rim of tinned iron to which a disk of fine-mesh monel metal gauze is soldered, a lead bar across the top gives sufficient weight.

Edison Capsules. These have been used to allow the sojourn of bacteria within the animal body without their coming into contact with the cells of the tissues. Various

and in one end there is fixed a small piece of thin glass tubing by gently heating the glass and inserting it. The tube becomes fixed when quite cold, and the junction is then painted round with collodion, which is allowed to dry thoroughly. The bore of the tubing is cleared of any obstructing gelatin, and the whole capsule is dipped into a solution of collodion so as to coat it completely. The collodion is allowed to dry, and the coating is repeated, it is also advisable to strengthen the layer by further painting it at the extremity and at the junction. The interior of the capsule is then filled with water by a

quantity of the broth is removed, and the contents are inoculated with the particular organism to be studied, or an emulsion is added. The glass tubing is seized in sterile forceps, and is sealed off in a small flame a short distance above the junction. The closed sac ought then to be placed in a tube of sterile broth to test its impermeability. The result is satisfactory if no growth occurs in the surrounding medium. The sac with its contents can now be transferred to the peritoneal cavity of an animal.

Autopsies on Animals after Inoculation

The examination should be made as soon as possible after death, it is preferable to kill the animal when it shows serious signs of illness. The surface of the animal should be soaked in 5 per cent. lysol, or in corrosive sublimate (1 : 1,000). This not only to a certain extent disinfects the skin, but, what is more important, prevents hairs which might be infected with pathogenic products from getting into the air of the laboratory. The animal is stretched out and firmly fixed by stout pins to a wooden board which is laid in a shallow trough made of metal or earthenware. The instruments necessary are scalpels (with metal handles), dissecting forceps (fine and coarse), and scissors sterilised by boiling for five minutes. Several, at boiling, they may be placed between sterile

skull, etc., is to be opened. It is well to have sterile capillary pipettes stored in a sterile cylindrical glass vessel (Fig. 220), also some larger sterile glass pipettes and sterile deep Petri dishes for receiving tissues. The hair of the abdomen of the animal is removed. If peritor should be at hand. It is also necessary to examine the sternum should side

flanks the fluid being allowed to run up the tube and the ends sealed, a small quantity may be taken in a sterile pipette. If peritoneal fluid is not required, an incision is made from the episternum to the pubes, and the thorax and abdomen opened in the usual way.

examined heart's a cautery, make an incision in the middle of the pericardium.

To examine any organ properly, it is necessary to introduce a looped wire and (see Fig. 790). The

organs are being dealt with the operator should wear gloves, and in the case of rickettsial and virus infections (q.v.), the eyes should be protected.

Spontaneous Infections common in laboratory animals

Guinea-pigs *Pseudo-tuberculosis* due to *B. pseudotuberculosis* rodentium, which is usually characterised by tubercle-like nodules in the abdominal organs, especially the spleen and lymph glands of neck.

Mice and *B. coli* worm (faecal glands of neck).

Rabbits. *Pseudo-tuberculosis* of chronic type (see Guinea-pig). *Respiratory infections* due to *P. leptoseptica* (nasal catarrh, bronchitis, pneumonia, pleurisy, pericarditis).

Enteritis due to *Salmonella* organisms or coccidiosis. *Spirochaetosis* (chronic ulcerative lesions of oro-nasal and ano-genital regions). *Myxomatosis* *Parasitic mange* (ears). *Encephalitis* *Helminths* (cysts in omentum and liver)

Rats *Respiratory infections* due to *Streptobacillus moniliformis*, etc. *Enteritis* due to *Salmonella* organisms, especially Gærtner's bacillus. *Mange* (root of tail and tip of ear) *Helminths* (cysts in liver and mesentery) *Aseptic suppuration* in glands of neck

OBTAINING OF BLOOD, PATHOLOGICAL MATERIALS, ETC. FOR EXAMINATION¹

All specimens intended for bacteriological examination should be taken with such precautions as will reduce to a minimum the chances of contamination from external sources. At the same time admixture with antiseptics must be avoided. Specimens should be received directly into sterile vessels. If possible, these should at once be closed with suitable sterile stoppers, otherwise, a portion of the specimen should be transferred at once to sterile containers provided with a stopper. Containers for sputum, etc., to be examined for tubercle bacilli must not be closed with ordinary corks, as these may

and 'blackol' paper liners are cheap and should be renewed each time. In most cases the examination (making of cultures, films, animal inoculation) should be carried out soon after withdrawal of the material from the body, as many pathogenic organisms soon tend to die or, when present in mixtures, to be overgrown.

Removal of Blood-Samples from Rabbits. With proper care any amount of blood up to one-third of that contained in the body can be removed from the ear-vein of a rabbit. The animal, which must not be excited, is placed on a bench, and its body kept warm by being covered with a cloth. The ear near the root should be shaved over the marginal vein, the hairs on the edge of the ear should also be clipped short. It is best to have the ear dry, as the evaporation of fluid causes contraction of the vessels. In a great deal of hæmolytic work absolute sterility of the sample is not necessary, so that washing the ear is not required. When sterile blood is desired, the precautions detailed

waves of contraction of the ear vessels will be observed to occur, the passing off of which must be waited for, and from time to time the clot must be gently squeezed out of the opening in the vein with the flat side of the needle, or it may be necessary slightly to enlarge the opening, but rubbing of the area must be avoided. The blood should be allowed to clot completely, and then, by means of a sterile platinum wire, the clot should

watch the progress of an immunisation procedure by removing a sample of blood, e.g. 20 c.c. by this means, without the animal being killed.

This method can be applied in guinea-pigs, provided these be of fair size. Here successive samples of 2 c.c. can be obtained from the ear veins. Daily samples can thus be obtained from an animal. If care be taken not to make ragged openings in the

¹ A packet containing a pathological specimen may be sent by post provided that the regulations of the Postmaster-General are complied with. The packet must be clearly marked 'pathological specimen—fragile, with care' and be sent by letter post only. The specimen must be enclosed in a container which is surrounded by sufficient absorbent material (cotton wool, sawdust, etc.) to prevent any leakage in case of breakage. The packing material must also prevent the container from shifting about inside a stout case in which the whole is packed.

vein, often the simple removal of the previous scab will be followed by a free blood flow.

Blood may also be obtained from rabbits, guinea-pigs, and fowls by cardiac puncture (p. 746), or from the wing-vein in birds.

If it is desired to bleed the animal out it may be anaesthetised with ether and bled from the carotid artery. The blood is received into a sterile tube which has been rinsed out with saline to ensure retraction of the clot. The serum which has separated from the clot on standing for twenty-four hours in a cool place is stored in lengths of sterile quill *in a dry place, or in a water-bath at 50° C. or in a vacuum desiccator over a naphthalene flame. It is then added to the addition of the serum,*

Blood for Cultivation, Serum, etc. In the human subject this should be drawn from a vein. The skin of the arm at the bend of the elbow is cleansed with sterile gauze swabs soaked in alcohol or the mixture of brilliant green and crystal violet (p. 805); then a tourniquet, e.g. several turns of stout bandage or indiarubber gas-tubing, is applied round the upper arm about the middle of the biceps, or the hands of an assistant, so as to render the veins turgid, but not to obliterate the radial pulse. Congestion of the veins is aided by the patient opening and closing his hand vigorously; in difficult cases the arm may be immersed in a basin of warm water. (In stout subjects the vein may be palpable to the finger as an elastic cord when it cannot be seen.) The skin at the elbow is rendered tense from behind by the operator's left hand; the syringe, with needle attached, is held in the right hand and almost parallel with the patient's arm; then the needle, with the eye turned away from the skin, is inserted into a prominent vein. The needle being pointed, blood are drawn into the syringe by the plunger, or the needle is withdrawn and the blood, otherwise a

hæmatoma tends to form, thereafter the needle is withdrawn from the vein, and the contents of the syringe are at once emptied into a suitable receptacle, test-tube, flask, etc. (the mouth of which must be flamed when the plug is removed), which is then closed again immediately. The syringe and needle must be thoroughly washed out with antiseptic solution without delay. Where the specimen must be transported for a considerable distance the container should be of stout glass and closed by an indiarubber stopper. Alternatively, a 6 oz screw-capped bottle is used (A $\frac{1}{2}$ in hole is punched in the metal cap, a rubber washer is inserted, 100 c.c. fluid medium is placed in the bottle, the cap screwed down and the apparatus is sterilised. When cool, a

syringe the rubber is punctured with the needle through the cap, and the medium is drawn into the bottle, and its contents well mixed. The skin puncture may be sealed with gauze and collodion.

In making blood cultures in order to detect the presence of enteric bacilli 5 c.c. of blood are added to 10 c.c. of sterilised ox bile or 50 c.c. of a 0.5 per cent. solution of bile salts in 1 per cent. sodium citrate. In examining for other organisms, e.g. pneumococci, staphylococci, or streptococci, 5 or 10 c.c. of blood are added to a large quantity of fluid medium, (100 c.c. or more). It is advisable to add an anti-coagulant to the medium, (e.g. 0.1 per cent. of sodium citrate).

Under the above conditions, but heparin is more suitable, as it also inhibits anti-bacterial properties. According to the amount of heparin used, the medium is more or less suitable for growth of aerobic organisms in blood culture. It is advisable to use broth made from meat extract and peptone or from digest and in either case containing 1 in 100 of heparin per 100 c.c. (added to the medium).

Each should be cultured in 10 c.c. of Brewer's broth. The medium should be mixed with an equal volume of a solution of 0.1 per cent. of sodium citrate. The medium should be mixed with an equal volume of a solution of 0.1 per cent. of sodium citrate.

¹ Supplied by Marshall Leconte & Co., 32 Lynton Crescent, Ilford, Essex.

² Supplied by Viscose Development Co., Woldham Road, Bromley, Kent.

sodium chloride 6 grms in 1,000 c.c. of water which is distributed in tubes (all sterilised before use). Coagulation is thus prevented and large quantities of blood can then

be drawn off in 'all glass' type syringes (I.S.W.G., 18 to 20); the separate parts should be placed in cold water and sterilised

and kept closed until required. For the mode of use of the 'Balco' syringe see p. 803

Behring needle.¹ This is a complete apparatus, supplied sterile, for withdrawing a specimen of blood from a vein. For making blood cultures a form is provided containing suitable media with which the blood automatically mixes.

For serological tests another method is to insert a needle into the vein and to allow the blood to flow directly from it into the tube. When the serum is required, frothing of the blood must be avoided. Bacterial contamination of specimens for the Wassermann test may be prevented by adding one drop of 6.5 per cent. aqueous solution of sodium azide per c.c. to the separated serum.

Method of obtaining a small sample of blood serum. The circulation in the hand is

the tube so that the other end can then be sealed. In this way several c.c. of blood may be obtained. Separation of serum is aided by centrifuging the tube.

Cerebro-spinal Fluid. Lumbar puncture is carried out with a special needle fitted

is fully exposed. The skin over the lumbar region is then carefully cleansed and sterilised and the operator must observe strict aseptic precautions throughout. The spines of the lumbar vertebrae having been counted, the left thumb or forefinger is pressed into the space between the third and fourth or fourth and fifth spines in the middle line, the needle (with the stylette in place) is then inserted about 1 cm. to the right of the middle line at this level (or the needle may be inserted in the middle line) and pushed through the tissues, its course being directed slightly inwards and upwards till it enters the subdural space (about 5 to 6 cm. in the adult to 1.0 cm. in a small child). When this occurs, the stylette is withdrawn and fluid passes along the needle, sometimes actually

for some hours afterwards.

Fæces. Specimens intended for bacteriological examination should not be mixed with urine. For the detection of bacilli of the enteric and dysentery groups a loose motion must be obtained, and in the case of suspected carriers it may be necessary to administer a purgative, e.g. calomel. On the other hand, for the detection of cysts of *Entamoeba histolytica* portions of mucus from the surface of solid motions are selected

¹ Metal cases with screw lids to accommodate syringes are obtainable commercially.

² Supplied by Bayer Products Ltd., Africa House, Kingsway, London, W.C.2

Of a loose motion 1 c.c. is sufficient. The usual sterilised swab soaked in the faeces and replaced in its test-tube.

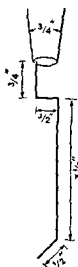


FIG. 225. Post-nasal swab (After Min Huh Bull, 1946, 5, 187)

most apparatus having been sterilised beforehand by autoclaving; or a 1 lb. clear glass jar with 3 in. neck and a screw lid with rubber washer, is very suitable for hospital use. Universal containers may also be used.

glass spec-
stouter th

organisms of the enteric and dysentery groups must be delayed for more than several hours after the

suppression of the specific organisms by *B. coli* which would otherwise occur.

Secretions from Throat, Nose, etc. Specimens of these secretions are obtained by rubbing the surface with a swab of cotton wool wrapped round the end of a strong wire. The swab is kept in a narrow test-tube of stout glass, and the other end of the wire is fixed in the cork stopper. The whole apparatus is sterilised in the autoclave and kept ready for use. In taking specimens from the throat it is important that no antiseptic should have been applied (e.g. as a gargle) for some hours before. A nasal swab should be mounted on a thinner wire, e.g. 1 S.W.G. 19.

Secretion from the Naso-pharynx. A specimen of pharyngeal mucus may be obtained by means of a swab of cotton wool on the end of a metal wire. The wire ought to be longer than that used in the case of a throat swab and bent near the extremity. A tongue depressor

is used, the wire is introduced into the mouth, and passed up behind the soft palate and then brought into contact with the posterior pharyngeal wall. Care must be taken not to touch any part of the mucous membrane of the mouth. The best method, however, is by means of West's tube. This consists of a glass tube shaped like a catheter, in the interior of which is a thin wire bearing the swab, the latter being just within the end of the tube. The bend of the tube can be used to depress the tongue, or a depressor may be used, and when the tube is sufficiently introduced it is turned up behind the soft palate, and the end of the wire is pressed so as to protrude the swab, which is brought into contact with the pharyngeal wall. The swab is then drawn back into the tube and the tube is removed from the mouth. It is important to avoid contamination with mouth organisms, which tend to inhibit the growth of meningococci. Cultures for the latter must be made at once and placed in the incubator as quickly as possible, as cold has an injurious effect on the organisms. If the inoculations have been made at some distance from the laboratory, the plates should be carried in an apparatus with a hot-water jacket—a bag containing a hot-water bottle is often sufficient. If it is not practicable to make cultures at the time, the swab should be inserted into a small amount of blood agar at the foot of a stout test-tube, this may be kept warm in a pocket. Plates should be inoculated both from the swab and the blood agar.

Fig. 225 illustrates a post-nasal swab suitable for obtaining specimens from children aged six months to five years for the diagnosis of whooping-cough (Tarr). No 16 S.W.G. brass wire is used and the whole apparatus, mounted on a cork, is enclosed in a stout glass tube and sterilised.

Urine. Care must be taken to prevent the contamination of the urine by extraneous organisms. In the male, specimens withdrawn by a sterile catheter into a sterile vessel are preferable, but it is often sufficient to wash thoroughly the glans penis and the meatus with 1:1,000 corrosive sublimate—the lips of the meatus being everted for more thorough cleansing; the urine is then passed into a series of sterile containers, the first of which is rejected in case contamination has occurred. In the female, after similar precautions as regards external cleansing, a sterile catheter must be used. The specimen should be centrifuged and films and cultures made from the sediment. In examining for tubercle bacilli it may be advisable to collect the twenty-four hours' urine in the ordinary way and



FIG. 226. Test-tube and pipette arranged for obtaining fluids containing bacteria

to remove some of the sediment which has settled and concentrate the latter in the centrifuge

Post-mortem Material. Since all surfaces, instruments, etc., in the post-mortem room are likely to be grossly contaminated, the first care should be given to obtaining specimens of body fluids where bacteriological examination is important. Blood should be

cotton wool which is inserted before sterilising

Solid organs to be examined should, if possible, be obtained whole. They may be treated in one of two ways. (1) The surface over one part at least 1 in. broad is seared with a cautery heated to dull red heat. All superficial organisms are thus killed. An incision is made in this seared zone with a sterile scalpel, and small quantities of the juice are removed by a platinum spud to make cover-glass preparations and plate or smear cultures, etc. (a piece of quill tube drawn out in the flame till slightly narrowed at one end which is then broken across so as to leave a jagged edge, is a good substitute for the spud) (2) An alternative method is as follows. The surface is sterilised by soaking it well with 1 : 1,000 corrosive sublimate for half an hour. It is then dried, and the capsule of the organ is cut through with a sterile knife, the incision being further deepened by tearing. In this way an uncontaminated surface is obtained.

The clinical history of the case will often suggest what the procedure ought to be in examination. It should be remembered that organisms may occur in the tissues of cadavers apart from disease processes, owing to invasion *ante* or *post mortem*. Among those specially likely to be found are *B. coli*, staphylococci, non-hæmolytic streptococci, and *B. Welchii*. The organs most likely to be infected in this way are the lungs and then, in descending order of frequency, kidneys, liver, spleen, and heart blood (Burn). In bodies placed at 10° C. soon after death no marked difference has been found as regards the presence of bacteria after one hour and forty-eight hours.

V

EXAMINATIONS IN CONTROL OF CHEMOTHERAPY WITH PENICILLIN, SULPHONAMIDES, AND STREPTOMYCIN

Estimation of Penicillin in the Blood, Cerebro-spinal Fluid, etc., and Urine. In the control of systemic treatment with penicillin it is essential to know the rate of absorption of the drug occurs. the plasma or serum. According to the method of

the method is that used for estimating the effect of a slowly-acting antiseptic in a fluid medium. Under aseptic conditions 0.5 c.c. amounts of doubling dilutions of the patient's serum (freed from red blood corpuscles by centrifuging) are placed in a series of 3 in. by $\frac{1}{2}$ in. test-tubes plugged with cotton wool, i.e. undiluted, 1 in 2, 1 in 4 to 1 in 64, etc., the diluent being broth. Then each tube is inoculated with a uniform amount of staphylococcus, say 0.05 c.c. of a 1 in 300 dilution in broth of a twenty-four-hours' broth culture (the strains recommended for use are N.C.T.C. 6571 A or 6718). The tubes are incubated at 37° C. and the results read after twenty-four and forty-eight hours. Growth is shown by the development of turbidity, while the sterile tubes remain clear. It is advisable, however, at least until the observer has gained experience, to check the results by making subcultures, since accidental contaminations may occur; also partial inhibition of growth, short of sterility, may lead to only a slight cloudiness which is readily overlooked. The subculture consists of a single stroke with a loopful of each mixture; thus a 4-in. Petri plate of agar will accommodate a considerable number. By comparison with the control series the concentration of penicillin in the serum may be expressed in terms of units. It should be noted that any slowly acting antiseptic is liable to give varying results in duplicate tests of this kind; therefore if a high degree of accuracy is desired, multiple estimations should be made with the same specimen of serum. The pH of the medium must be standardised, penicillin being acidic is diminished in its antibacterial activity by increase of pH. Human serum has a certain destructive effect on penicillin, therefore tests should be made as soon as possible after withdrawal of the blood or, if there must be an interval up to twenty-four hours, the serum should be kept in the ice-chest. The penicillin-content of cerebro-spinal fluid, exudates, etc., may be tested in the same way. The standard culture should be subcultured once a month and the culture kept in the ice-chest. From time to time plating should be carried out and the next subculture made from a mixture of several typical smooth colonies.

Urine may be tested like serum by the dilution method. It must first of all be sterilised by filtration, also, as its content in penicillin is likely to be high during the administration of the drug (unless there is infection of the urinary tract with organisms which produce penicillinase), it is usual to prepare dilutions in broth of 1 in 100, 1 in 200, etc. The examination of the urine is chiefly of value in checking the permeability of the kidney.

Other organisms have been used instead of the staphylococcus for such tests, e.g. sensitive streptococci or sporing Gram-positive aerobic bacilli.

Another method consists in using Petri plates into which has been poured agar inoculated in the melted state with 1.0 to 0.1 c.c. of a twenty-four-hours' broth culture of the standard staphylococcus. The plates ought to be firmly set, and may be kept in the ice-chest for twenty-four hours before use, as little growth occurs under these conditions. Then sterile 12-mm.-long cylinders of glass tubing, 8 mm. in diameter and 1 mm. thick, with smooth ends (or similar tubes of stainless steel) are warmed in the Bunsen flame and placed on end equidistant on the surface of the medium (six for a 4-in. plate). They should be made sufficiently warm to cause the agar to adhere when the plate is inverted, but not hot enough to melt the medium, thus a series of cups is formed. The plates must be at once returned to the ice-chest till the cups are to be filled with the test fluids. For use the cups are practically filled by means of a pipette with serial dilutions of the specimen.

The results are horizontal. Then ascertain in units per c.c. multiplied by the dilution factor. The results are stated to be most reliable when the values for the specimen coincide with points corresponding to between 0.3 and 1.4 units of penicillin per c.c.

Micro-methods involving the use of slide cells or capillary tubes have been described by Fleming, in which the penicillin content is estimated according to the inhibition of hæmolysis produced by the patient's serum, etc., in a mixture of broth and human group O blood (deprived of living leucocytes) inoculated with a hæmolytic streptococcus.

Estimation of Sensitiveness of Organisms to Penicillin and to Sulphonamides. The prospect of treatment with penicillin proving effective in the individual patient depends on whether the infecting organisms are sensitive to the drug. This may be determined by inoculating medium containing penicillin and ascertaining the behaviour of the organisms in comparison with a standard culture. The test may be carried out in the same way as that described above for the control in fluid medium, except that the organisms under investigation and the standard culture are compared in parallel series. A rapid method apparently satisfactory for practical purposes is that recommended by Fleming. Towards the side of a plate of agar or blood agar (20 c.c. of medium for a 4 in. plate) a rectangular strip of the medium about $\frac{1}{2}$ in. wide is removed, so that a ditch is left. The cut edges of the ditch are then sealed to the glass by touching with a warm metal rod (this prevents fluid from passing between the medium and the glass). On the surface of the medium a stroke culture is then made of the organism to be tested, which is at right angles to the ditch and starts from its edge—several different cultures including the control standard staphylococcus may be tested on one plate at the same time. Finally the ditch is filled with a solution of penicillin containing 1 to 10 units per c.c. The plate is kept at 37° C. for eighteen to twenty-four hours or longer, then the

(*vide infra*)

In the case of sulphonamide drugs a similar ditch is cut across the centre of the

twenty-four hours' culture of streptococci in serum broth, since the inhibitory effect of sulphonamides is most apparent with small numbers of organisms. After incubation the lengths of the zones of inhibition are compared as in the case of penicillin.

Estimation of the Amount of Streptomycin Contained in Serum. This may be carried out as for penicillin. A strain of Friedländer's bacillus (*Klebsiella pneumoniae* 41)

is more sensitive to penicillin. According to Mav *et al.*, the result depends on the size of the inoculum, access of oxygen, and the use of distilled water. A mixture of one volume of a 24-hours' broth culture of the organism and one volume of a 24-hours' saline culture of patient's serum (previously heated at 56° C. for 30 minutes) are mixed and distributed either into small test-tubes (0.5 c.c.) or capillary tubes (2.5 cm. column of fluid)—the latter being left open and fixed horizontal with plasticine. After 24 hours at 37° C. the results are read. Growth of the organisms, which will occur in concentrations of streptomycin under 1 in 4 to 6 millions, is shown by the transparent red fluid becoming yellow, opaque, and clotted. A control series is set up with human serum containing a known concentration of streptomycin (e.g. 1 in 1 million). By comparison with this the amount in the patient's serum is estimated.

Test of Sensitiveness of a Culture of the Tubercle Bacillus to Streptomycin. A homo-

Dubos *et al.* (*vide infra*) in a Bijou screw-capped bottle. After 7 to 10 days at 37° C, 0.05 c.c. of the growth is used to inoculate a bottle of the same mixture, which should show good growth after 7 to 10 days' incubation. The culture, diluted if required, should match Brown's opacity tube No. 2; it is then diluted 10 times further for use.

A similar series is prepared with a standard virulent human strain of *B. tuberculosis* (H37 Rv of the U.S.A. Depot of Standard Cultures). This is maintained by triplicate subcultures made at 3-weekly intervals in Proskauer and Beck's medium (KH_2PO_4 5 grms, asparagine 5 grms, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6 gm, magnesium citrate 2.5 grms, glycerol 20 c.c., water to 1,000 c.c., adjusted to pH 7.8 with 40 per cent solution of NaOH, about 3.5 c.c. being needed, and autoclaved at 10 lb pressure for 20 minutes, after which the pH should be about 7.4). A portion of the surface growth is used to inoculate a bottle of Tween-albumin medium and a subculture from this (as above) employed for testing sensitivity. A comparison of the concentrations of streptomycin which inhibit growth of the standard organism and that under test gives a measure of the relative sensitiveness of the latter.

(Special precautions must be taken in pipetting suspensions of living tubercle bacilli in order to avoid dispersal of the organisms in the air.)

Tween-albumin medium for submerged-dispersed growth of tubercle bacilli (Dubos and Middlebrook). Dissolve KH_2PO_4 1 gm, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.3 grms, and asparagine 1 to 2 grms in 100 c.c. water by heat, then add 850 c.c. water and 20 to 40 c.c. autocla-

Fa
is
of each of the following— ZnSO_4 0.01 per cent, CuSO_4 0.01 per cent; then adjust the solution to pH 6.5 to 6.8. To 90 c.c. of this mixture add 0.5 c.c. of a 10 per cent aqueous solution of Tween-80 (which is kept in the refrigerator and must not be used when more than one month old), sterilise in the autoclave and add 10 c.c. of 5 per cent aqueous solution of albumin (Armour's 'bovine plasma fraction V') previously sterilised by filtration, preferably through a sintered glass or porcelain filter, and been sterilised in the autoclave. Gl
substitute for the albumin may be
to pH 2.0 by addin
minutes, centrifuge
precipitate is removed

Medium used by the F ... *committee of the Streptomycin*
Trials Committee, Medical
of Dubos and Davis (p. 2-
citrate 1.5 grms, MgSO_4
(*vide supra*), then 5 c.c. 10 per cent solution of Tween-80, ... The medium, the
acid hydrolysate (p. 738) and distilled water to 1000 c.c. are added. The medium, the
pH of which should be 7.2, is distributed in amounts of 2.5 c.c. in Bijou screw-capped
bottles and autoclaved at 10 lb pressure for 10 minutes. Before use 0.1 c.c. of 9 per
cent solution of bovine albumin (*vide supra*) sterilised by filtration through a disk of
Seitz type, is added to each bottle. (The medium is supplied by Dr J. E. McCartney,
Southern Group Laboratory, Park Hospital, Hither Green, London, S.E. 13)

¹ Obtainable from Messrs Honeywell and Stein, Ltd., 21 St. James's Square, London.
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VI

METHODS OF DEMONSTRATING FILTERABLE VIRUSES

FILTRATION

In order to obviate fallacious results rigid precautions must be exercised in attempting to demonstrate the capacity of viruses to pass through filters which retain the ordinary bacteria (p. 768). The filtration should be carried out within the minimum time, but the most important precaution is that the filtration should be as slight as possible, 50 mm.

prodigiosus to the material before filtration and test the filtrate for sterility by the usual culture methods—this organism is readily recognised by the pink colour of its colonies growing at room temperature. In any case the bacteriological sterility of the filtrate must be controlled by making cultures under both aerobic and anaerobic conditions. The demonstration of the virus will depend finally on the experimental production of the characteristic disease in susceptible animals (or, in some cases, human volunteers) by inoculation of the filtrate obtained from material likely to contain the virus (e.g. blood serum, nasal and throat secretions, tissue emulsions, inflammatory exudates). To establish the occurrence of propagation on the part of the virus, passage through a series of susceptible animals is carried out after the first experimental results are obtained, cultivation in the chorio-allantoic membrane, etc. of chick embryos may also be combined with this. In preliminary experiments one of the coarser filters is used, e.g. a Berkefeld V or Chamberland L₂. In further tests, finer filters may be employed to estimate the degree of filterability of the virus. With a view to determining the size of virus particles, methods of 'ultra filtration' have been applied in which the filter membrane consists of a thin layer of collodion. According to their mode of preparation the permeability of such membranes may be graded (*vide* p. 523), the permeability also depends on the filtration pressure employed. Collodion membranes are now chiefly used for work on viruses.¹ A difficulty which attends the interpretation of the results of such experiments is that the virus may be attached to other particles, and so its retention by the filter may be due to the characters of these. Further, absorption of the virus by the substance of the filter, and not the size of the particles, may account for its retention (The distribution of a virus when a suspension of material containing it is centrifuged may likewise depend on the size of the particles to which it is attached.)

INCLUSION BODIES

Staining. Inclusion bodies are usually acidophile and less frequently basophile. Dilute Giemsa's stain, used as in staining elementary bodies (*vide infra*), is suitable for

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70 per

cent alcohol), the differentiation being controlled under the microscope, the sections are then dehydrated and mounted. Tissues fixed in formalin are not suited for staining by the above method, but good results may be got by substituting aniline blue or soluble blue (C.I. No. 707) for methyl blue in the stain, allowing it to act for five to ten minutes at 40° C. on the section and differentiating in alcohol to which a trace of alkali has been added (Hutchinson).

¹ Collodion 'gradocol' membranes are obtainable commercially

Ford's modification of Mann's stain. Sections are stained as above for three hours at 37° C, then treated with pure formalin for five seconds, washed in water and differentiated as in the original process. This method has been found particularly valuable in demonstrating the Negri bodies of rabies.

ELEMENTARY BODIES

Demonstration by ordinary Microscopic Methods. The occurrence of these minute structures is of importance in the recognition of certain virus diseases; but the usefulness of the ordinary microscope for this purpose has been somewhat overlooked. The limit of visibility attainable with white light is stated to be 0.074μ and with green light 0.0673μ (Coles). The macula is more sensitive to green light and the additional contrast which is imparted enables smaller objects to be more easily detected. It must be borne

in mind that the investigator as well as his visual acuity and the optical equipment.

Optical equipment. It is important both to have suitable equipment and to make correct use of it. As regards the microscope and its accessories, the following points are important. The stand must be of good quality with a sensitive fine adjustment, free from 'back lash'; there should be a smooth-working mechanical stage which will enable the object to be kept in view under the 2 mm. objective while the preparation is being traversed. An achromatic substage condenser is required, preferably one suitable for use with oil-immersion, the mounting should have rack and pinion focusing and also

the lamp should be fitted with a condenser and an iris diaphragm for dark-ground illumination. The stage should be fitted with a condenser and an iris diaphragm for dark-ground illumination.

on slides 1-1 in. The surfaces under the slide into close apposition. The preparation should be viewed first by using the low-power dry objective to ascertain the types of cells present, and then with the

scrutinised, first with the $\times 10$ and then the $\times 15$ ocular, the light adjusted continuously used and the height of the substage condenser varied slightly, so as to focus accurately the light upon individual particles. The diaphragm may be slightly closed in

in elementary bodies. The following are included in every series, and morphology determined by dark-field illumination. The following are removed from

seen in the form of small, round, or oval, elementary bodies. The means of animal origin of the chorion, and others, can be determined by their fluorescence. Staining is effected by fifteen seconds with a 0.1 per cent. solution of the dye in 2 per cent. aqueous distilled

water, (which keeps in the dark for a few days) followed by rinsing with distilled water (Hagemann).

Elementary bodies are usually most abundant in early lesions e.g. in the pox diseases, in scrapings from papules, or in the contents of vesicles before they have begun to show turbidity. Craigie obtained them in third day lesions of vaccinia in rabbits by lightly cleansing the skin lesions with distilled water, then placing a drop on the area and through this gently scraping the lesion with a scalpel. The fluid was used to make thin films on slides.

Staining Methods. *Giemsa's solution* (p. 789) diluted 1 in 10 with distilled water and applied for twelve to twenty-four hours to films fixed with methyl alcohol has been extensively employed. The elementary bodies of vaccinia, psittacosis herpes etc. may all be stained in this way. The principal disadvantages of the method, however, are its slowness and the tendency for the stain to form deposits. Further, different samples of the stain vary considerably in their staining affinities.

Paschen's Stain. For speed and reliability this method is unrivalled. Films from infected material are made on slides, allowed to dry; placed in distilled water for three minutes; dried in air; fixed in absolute alcohol for five minutes, dried in air, treated with Löffler's flagella mordant (tannic acid, 20 per cent aqueous solution, 100 c.c., ferrous sulphate, saturated solution, 50 c.c., saturated alcoholic solution of basic fuchsin, 10 c.c.) which should be gently heated for one minute and allowed to act for ten minutes, washed well in distilled water and stained for ten minutes with carbol-fuchsin solution diluted 1 in 20 (with the application of heat for one minute). Excess of fuchsin may be removed by treatment with water slightly acidulated. The preparation should thereafter be rapidly washed in water, dried by blotting, and mounted. Elementary bodies have a deep red colour.

Methyl violet (Gutstein's method). The film is dried at room temperature or 37°C followed by distilled water. The slide is placed in a dry Petri dish and filter paper moistened with methyl violet in distilled water is placed over it. The slide is then placed at 37°C for twenty to thirty minutes. Rinse in distilled water, dry and mount. Elementary bodies are stained violet.

Victoria blue (Gutstein's method). A film prepared and fixed as for methyl violet staining (*vide supra*) is laid film-side downward in a Petri dish, being supported by two pieces of capillary glass tube. A freshly made and filtered mixture of equal volumes of Victoria blue 4R (C.I. No. 690) 1 gm. dissolved in absolute alcohol 10 c.c. and distilled water 90 c.c., and of 0.02 per cent aqueous KOH solution is run under the slide. The dish is covered and left overnight at room temperature, then the slide is rinsed with distilled water, dried quickly in the air, and mounted. The elementary bodies are stained dark blue.

Castaneda's method. To 20 c.c. of phosphate buffer solution of pH 7.6 containing 0.2 per cent formalin, add 10 drops of 1 per cent aqueous methylene blue solution and 1 c.c. formalin. Let this mixture flow over an unfixed film on a slide and stain for two to three minutes. Wash in running water for thirty seconds, counterstain for one to two seconds with aqueous solution of safranin (0.2 per cent safranin O, 1 part, plus 0.1 per cent acetic acid 3 parts, has been recommended), wash in running water and dry. Elementary bodies are stained deep blue, likewise the Rickettsiae of typhus, other structures are stained pink by the safranin.

Suspensions for Agglutination, etc. Suspensions of elementary bodies of vaccinia and fowlpox may be obtained practically free from accompanying tissue products as follows (Ledingham). Early lesions are thoroughly triturated in a mortar with distilled water, and the resulting suspension along with at least an equal volume of ether is shaken frequently over twenty-four hours and then centrifuged at 9,000 r.p.m. The supernatant ether and a middle layer are removed and then the remaining fluid with any deposit resuspended in it, is centrifuged again for at least one hour. The resulting sediment is resuspended in saline containing 0.25 per cent formalin and subjected to fractional centrifugation until a suspension of pure elementary bodies is obtained, as judged by microscopic examination of stained films. In Craigie's procedure the affected skin from rabbits three days after light vaccination on the back and sides with diluted lapine, is pinned on a board, lightly cleansed with distilled water and scraped with a scalpel into two successive 10 c.c. amounts of distilled water buffered at pH 7.0 with M/250 citric acid and disodium hydrogen phosphate. The suspension is centrifuged for five minutes at 1,500 r.p.m., the supernatant removed and the deposit resuspended in 10 c.c. of the same buffered water and again centrifuged, then the two supernatants are pooled. This crude suspension is spun in an angle centrifuge for an hour at 3,500 r.p.m. in flat tubes of 4 to 5 mm. internal width, the supernatants rejected and the tubes inverted on filter-paper to drain, after this the deposits are resuspended in the original volume of buffered water,

the centrifuging, etc., repeated and the deposit finally dispersed thoroughly in the original volume of buffered water. This suspension is spun in an ordinary centrifuge to remove gross particles. For serological tests 0.25 per cent formalin is added. Amies obtained the elementary bodies of varicella which must be clear, and must prevent coagulation (e.g. the tubes of 5 mm diameter for thirty minutes at 10,000 to 12,000 r.p.m., the supernatant rejected and the sediment resuspended in formal-saline as above; before use this is centrifuged for a short time to remove any debris).

CULTIVATION

Cultivation in the Developing Egg. The methods used by different workers vary in detail. The following procedures are recommended by Beveridge and Burnet. The eggs should be from one breed of hens and of standard size—not less than 53 grms weight.

The eggs making examination preferably the eggs should be incubated for 24 to 36 hours before use.

Incubation at different ages. A 100-watt lamp housed in a small wooden box with an egg-shaped opening in the side, is used—during the examination all stray light is excluded. The embryo must be alive, this is shown by its spontaneous movements (which are most marked when the egg is newly out of the incubator and warm) and the well-defined shadow of blood vessels. The position of the air space is noted (usually at the blunt end of the egg), and its extent marked by a pencil. The orientation of the embryo is determined by the convergence of two lines drawn from the air space to the embryo, regards everything.

The egg is supported at three points on a stand the basis of which is an isosceles triangle with sides 4 in. long and the base 2 in. Trays with similar openings are used for holding inoculated eggs at 35° C in the incubator, which is of the ordinary bacteriological type.

For drilling the shells a dental engine is used or a hand drill, vulcanite carborundum disks (e.g. Vulcarbo No. 8) are satisfactory, but for simple inoculation a round steel burr is convenient. The embryonic membranes must not be damaged, hence visible bleeding ought not to occur—any hæmorrhage tends to cause non-specific lesions. A triangular cutting-edge needle mounted in a handle is used for removing the shell over the drilled area, puncturing the shell membrane, etc.

Chorio-allantoic membrane. When it is desired to produce focal lesions, eggs incubated for 12 to 14 hours are used.

The inoculum is applied to the groove over the air space, which is drawn in the shell membrane and the drop of saline is drawn in. The cut in the shell membrane is now extended and a piece is reflected or cut away to make an opening for inoculation. 0.05 c.c. of the inoculum is deposited with a capillary pipette held vertically, neither shell nor membrane touched. The opening in the shell is sealed, e.g. by a slightly boiling water dipped into 10 per cent egg white. The air space is left open. The post-inoculation. It is incubated as a rule for two

to three days. An alternative method which causes minimal traumatic damage, but requires more skill, is the following. By means of a side to-side movement of the rotating disk an oval opening 2 mm. by 3 mm. is made in the shell and further procedures carried out as soon as

possible, and at latest within an hour. Sterile melted paraffin wax is painted round the aperture (the shell membrane must not be touched), then a minute drop of liquid paraffin is applied to this membrane, so as to render it transparent and non-wettable, 0.05 c.c. of inoculum is deposited on the exposed shell membrane and a puncture is made through the drop by means of a glass needle with a fused sphere $\frac{1}{8}$ mm. in diameter at the tip, the point being moved down and at the same time dragged towards the operator. The inoculum is drawn in and distributed over the chorio-allantois by making suction at the opening into the air sac.

Examination The egg is placed on a pad of cotton wool moist with antiseptic solution, which is moulded for the purpose, and placed in an open Petri dish. Sealing material is removed and the shell rapidly flamed. The shell is broken off to the level of the fallen chorio-allantois, which is then pulled away slightly with forceps and cut off with scissors beyond the limits of the artificial air space. Thus the whole inoculated area is removed. For counting foci, the membrane is washed with 10 per cent. formal-saline, spread on a glass plate, and examined against a dark background with a hand lens or plate-counting microscope. If the virus is required as an inoculum or vaccine, formalin should be omitted. For histological examination the membrane should be pinned out on a board and fixed.

Material to be used for inoculating animals or further eggs is rubbed up in a small amount of broth in a stout test-tube with a glass rod. An estimate of the amount of virus present may be made when it is of a species which produces lesions in the chorio-allantoic membrane. The affected membrane is well ground in a mortar with fine quartz sand, then broth is added and the mixture is centrifuged at low speed to remove sand and tissue particles. Serial dilutions of the supernatant fluid are used to inoculate eggs, a fixed volume being employed. Where discrete plaques are produced the number of these is counted, the result thus giving an indication of the number of elementary bodies or clusters of these present in the inoculum. It was estimated by Burnet and Bull in the case of a strain of influenza virus that at least five virus particles were required on an average to produce one focus, since the intravenous route was five to ten times more sensitive than the chorio-allantoic.

Allantoic inoculation Eggs which have been incubated ten to eleven days are marked at a point where the chorio-allantois is well formed, but free from large blood vessels. A groove 3 mm. by 1 mm. is drilled through the shell without damage to the shell membrane. The egg is placed on a stand with the groove up. Melted paraffin wax is

incubation for testing for hæmagglutinin, by opening the inoculation aperture with the heated point of forceps and introducing a capillary pipette with a short conical end.

Amniotic inoculation is of special value for influenza viruses. In general 11- to 13 day embryos are the most suitable. On candling the egg an arrow is pencilled on the shell close to the air space where the shadow of the freely movable allantoic vein is seen (or, failing this, at the point of convergence of two large veins). Then the egg being held with the blunt end (air space) towards the operator, a mark is made half an inch clockwise from the arrow point and on the largest diameter of the egg. At this site an

Then the chorio-allantoic membrane is made to recede ('dropped') as described above, except that a drop of saline is not required. Thereafter all of the shell within the oval is removed and all exposed shell membrane cut away. The embryo usually lies directly below the opening. A piece of chorio-allantois free from large blood vessels is grasped with fine curve-pointed forceps and a cut made 0.5 cm. long without damaging the amnion underneath. The latter is grasped taut and pierced by a fine-pointed Pasteur pipette fitted with a rubber teat and with its end bevelled on a carborundum stone (the pipette has been filled beforehand with 0.05 to 0.25 c.c. of the inoculum and then

about 0.05 c.c. of air drawn up). On pressing the teat of the pipette the bubble of air is first ejected and its position shows if the amniotic sac has been entered; if so, the

This permits removal of the cover-glass for daily examinations, the experiment usually being terminated after 3 to 5 days' further incubation. The shell is now broken off down to the level of the chorio-allantois, which is cut away with scissors and the allantoic fluid drained off. The amnion is then grasped with forceps, a small incision made in it with scissors and the fluid drawn into a Pasteur pipette. (Normally the volume is 0.6 to 5 c.c. and up to the 13th to 15th day it is clear and watery; in influenza virus infection there may be almost none). The embryo is then removed to a Petri dish. The lungs provide a rich source of virus.

Yolk-sac inocul.

broad drill-cut ab-
being left intact
of 20 or 21 I.S.W.G. inserted for about 3 cm. in the long axis of the egg, i.e. to just beyond its centre, and 0.25 to 1 c.c. injected. The hole may be closed with paraffin wax. The egg is candled twice daily and as soon as the embryo is dead the yolk is removed. This is done by placing the egg vertically blunt end up, and breaking away the shell over the air

minutes and placed in a sterile tube.

Where the yolk fluid is a satisfactory source of virus, e.g. in psittacosis on the third day of incubation, it is sufficient to place the egg air space up and insert a hypodermic needle attached to a syringe for about three-quarters of an inch; thus 1 c.c. or more of fluid may be got.

For details of other methods of inoculating eggs the original descriptions of Beveridge and Burnet should be consulted.

Cultivation *in vitro* in Minced Chick Embryo. According to Li and Rivers, embryos nine to twelve days old are removed from carefully sterilised egg shells with aseptic precautions, the eyes being discarded, and then finely minced in a sterile glass receptacle with sharp scissors; 5 c.c. of Tyrode's solution are added to 1 gm. of the mince (or less as excess of tissue may hinder proliferation of virus) and placed in a 50 c.c. Erlenmeyer flask. The mince is then inoculated with virus suspension, say 0.25 c.c. to prevent evaporation, e.g. four or five days

freshly distilled water in perfectly clean glass vessels should be used for the preparation of Tyrode's solution.

Cultivation in the presence of surviving tissue cells (Maitland and Maitland). The cultivation of vaccinia virus in a medium containing the fresh kidney tissue of the rabbit or hen is described on p. 562.

Cultivation in tissue cultures. Growths of epithelia, and fibroblast cells have been proved to be susceptible to infection by certain viruses. The methods employed are too complicated for detailed description here and the reader is referred to the original literature on this subject (Bland and Canti, Rivers, Haagen and Muckenfuss, Rhodes and van Rooyen).

PRESERVATION

Many viruses remain alive for long periods in material which is kept frozen, preferably on 'dry ice' (solid carbon dioxide) at -76°C . Certain viruses present in animal tissues, etc., can be preserved for considerable periods in 40 per cent glycerol made up in physiological saline, especially when kept in the ice-chest. The most effective method is rapid desiccation as in the preservation of complement (the lyophilic process, vide p. 770) and maintenance *in vacuo* at low temperatures. The tissue should be finely divided by mincing with scissors before desiccation.

HÆMACCUTININ OF INFLUENZA VIRUS

Titration (Salk's method) 10 c.c. of fowl's blood (p. 803) are mixed with 2 c.c. 5 per cent. sodium citrate solution and centrifuged. The sediment is washed three times with 5 to 10 volumes of saline. A 10 per cent stock suspension of the packed sediment is made, which keeps for a week at 4° C.¹, this is diluted just before use to yield a 0.25 per cent. suspension (to ensure a standard suspension its hæmoglobin content should be estimated by a colorimeter). A series of 0.5 c.c. amounts of doubling dilutions of purified virus (*vide infra*) ranging from 1 in 10 to 1 in 5,120, are measured into 7.5 by 1.0 cm. (internal diameter) test-tubes with rounded bases (a fresh pipette is used for each dilution), and to each 0.5 c.c. of blood suspension is added; a control tube contains red cells and saline without virus. The tubes are well shaken, set aside at room temperature and the results read after 1½ to 2 hours, by viewing the tubes from beneath, either directly or by setting them on a glass table and adjusting a mirror underneath. Unagglutinated red cells, as in the control, settle to form a small compact red disk; full agglutination is shown by a salmon-pink film uniformly covering the rounded base of the

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raised when the red cells are diminished. Temperature also has an effect, at 2° C. agglutination occurs more slowly but to a higher titre than at room temperature, while at 35° C. the virus dissociates from the red cells. When kept sterile at 4° C. the virus in the allantoic fluid remains unchanged for several months.

Antiagglutinin of serum For titration of antibodies which neutralise the agglutinating action of the virus, it is best to use the latter partially purified as follows. Virus containing allantoic fluid is secured by inoculating infective fluid into the allantoic sac of fertile hens' eggs which have been incubated for eleven or twelve days, after forty-eight hours' further incubation, the shell over the natural air space is removed and both the chorio-allantoic membrane and the amnion along with their blood vessels are torn. Admixture of the cavity fluids with the embryo's blood is secured by rotating the egg, the mixture is then removed with a syringe to a container, several eggs being treated thus to secure sufficient material. The container is immersed in water at 4° to 6° C. The red cells, which agglutinate rapidly, are centrifuged out, care being taken to avoid rise of temperature, the supernatant is discarded and the sediment gently rinsed with saline at 4° C. Then one tenth of the original volume of saline is added and the temperature brought to 37° C. with gentle shaking, the mixture then being incubated for 2½ hours. The agglutinated cells disperse as the virus dissociates. Finally, the mixture is centrifuged, the supernatant, which is slightly opalescent and pinkish, and infectivity remain un-

estimated by setting up with the red cell suspension two series of doubling dilutions, starting respectively at 1 in 20 and 1 in 30, the two results being averaged. Just before testing the sera the virus is diluted to half titre (other workers prefer 2 or 4 times as much virus). Each serum is diluted 1 in 8 (0.2 c.c. serum—previously heated at 55° C. for 30 minutes—

1 c.c. of 1 in 10,240 is required (10 tubes). To each tube
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ion, and

¹ Preservation of red blood corpuscles by Alsever's method is satisfactory. To washed red cells add an equal volume of the following solution: 2.05 grms. dextrose, 0.8 grm. sodium citrate, 0.42 grm. sodium chloride, 0.055 grm. citric acid, water to 100 c.c. (sterilised in the autoclave, final pH=6.1). Guinea-pig corpuscles kept in this solution show no lysis after 3 weeks or longer. The blood is washed thrice with saline before use. The reagent may be added to red corpuscles in serum or plasma (see Whitman, *J. Immunol.*, 1947, 56, 167).

in tube (2) 0.5 c.c. 1 in 8 dilution of normal serum—as a standard for comparison with the appearance in the tubes containing the highest amounts of the sera under test and (3) 0.5 c.c. saline as a standard for comparison with the tubes containing the higher dilutions of serum. The tubes are all well shaken and set aside at room temperature for $1\frac{1}{2}$ to 2 hours when results are read (*vide supra*). The highest dilution of a serum which completely prevents agglutination, is taken as its titre. The titre of normal serum is stated to be usually below 128, while that of a patient's serum may be 256 to 2,048; but these figures are only approximate, and depend on the strain of virus, the particular red cells used, etc. I have also at the same time a specimen of serum taken at the commencement of illness (which has been kept frozen) and one in convalescence from the same patient: a four-fold rise in titre of the latter may be accepted as significant of recent influenzal infection.

VII

BACTERIOPHAGE

Recovery from Faeces, Sewage, etc. Phages for various micro-organisms are frequent

for 12 to 24 hours, then passed through a bacterial filter (a Berkefeld or porcelain filter is preferable to a Seitz disk, as the latter may absorb a weak phage). To test the filtrate for phage action, stroke a loopful of each of a series of young broth cultures of likely organisms on an agar plate so as to secure broad bands of confluent growth, when the inocula have dried apply to each stroke a loopful of the filtrate, so as to form a drop, then incubate overnight. The appearance of an area of clearing at the site of the drop indicates the presence of phage active against the particular organism. When it is desired to obtain phage for a given organism, it is advisable to add a few drops of this culture to the mixture of broth with sewage etc. and then to proceed as described above. In this way enrichment of the required phage is favoured. In order to recover

to contain phage, it should also be plated and incubated. The presence of phage is suggested by the appearance of areas of clearing (plaques or 'nibbled' colonies), but careful examination may be needed not to miss these. To confirm this prepare young broth cultures from a considerable number of nibbled colonies of the organism. Incubate and filter, then test the filtrate for phage (*vide supra*).

When a phage has been demonstrated, the activity of the preparation can usually

culture is also made to detect plaques. The activity of the phage preparation is estimated quantitatively by the methods described on p. 604. A powerful sterile phage filtrate frequently retains its activity for periods of months or years if kept frozen or even at 4° C.

Isolation of Pure Strains of Phage. Proceed as described on p. 604.

It should be emphasised that in working with bacteriophages there is a great risk of accidental contamination with extraneous phages. Strict precautions are required

In the case of *B. typhosus*

vi phage II (strain 6)—of
with a strain of typhoid

so that in suitable concentrations it lysed actively the corresponding strain, but produced little or no lysis of

ed, some being
e above phage
ferences, which
have identified,
s—types A, B
D₁, D₆, D₈,
J, K, L (com-
type is highly
es may be very
D₁ is actively
(e) Type A is

highly susceptible to phages of all types and probably represents a rare variant of other types (which may develop either *in vivo* or *in vitro*); certain others, B and C especially, show a considerable degree of cross-sensitiveness. The number and size of plaques produced with the homologous or heterologous phages vary according to the type; thus with N, O, and T strains and the corresponding phages respectively small plaques are commonly produced.

The epidemiological value of typing lies in the information afforded as to the relations between cases, either sporadic or in an outbreak, and the carrier suspected to be the source of infection—a carrier may be absolved from responsibility for a given

able medium. Dorset's egg medium is recommended (this is prepared as described on p. 748, but with distilled water instead of digest broth, and without addition of any dye); cultures are incubated at 37° to 38° C. overnight and kept cool (preferably at 4° C.) in the dark; stock cultures should be transferred as seldom as possible. Make a subculture on Dorset's medium, and after incubation overnight plate it on agar (*vide infra*), so as to get separate colonies. After incubation overnight pick about a dozen of the most opaque, smooth colonies and with a mixture of these inoculate a further tube of Dorset's medium. As soon as this shows growth at 37° to 38° C. store it at 4° C. in the dark. The agar medium has the following composition—'Bacto' nutrient broth (dehydrated) of Difco Laboratories 20 grms., NaCl 7.5 grms., 'Bacto' agar 20 grms and distilled water 1,000 c.c. The final pH should be about 6.8 and must not be adjusted with alkali (tryptic digest agar may be used instead under certain controlled conditions). Each new batch of medium should be compared with the previous batch to ensure that N, O, and T strains when tested with all the phages yield the characteristic small plaques with the corresponding phages and show the typical behaviour with the rest. (2) Sterile concentrated fluid phages retain their activity unchanged for years at 4° C. and dilutions keep at least for several months. Since the preparation of specific phages is attended by the danger of accidental contamination by other types, it is advisable to use controlled products. These and also type cultures may be obtained from the Central Enteric Reference Laboratory, Public Health Laboratory Service, Colindale Avenue, London, N.W.9.

Preparatory to testing, make a subculture of the organism in Difco broth (or tryptic digest broth plus 1 per cent peptone); after 2 to 3 hours at 38° C. use it to inoculate richly the surface of a fairly thick layer of agar (25 to 30 c.c. of medium for a

reactions). Readings are made after 8 and 24 hours' incubation, against a dark background with oblique lighting, first of all with the naked eye and then, to confirm negative results, at a magnification of $\times 7$ to 10.

Certain other *Salmonella* organisms, e.g. *B. paratyphosus B*, may be typed on similar principles (Felix and Callow).

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(Note —*Zent. Bakt.* refers to Abt. I, Orig., unless otherwise specified)

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